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(54) Title: CD133 EPITOPES

(57) Abstract: An immunogen includes an isolated peptide that includes the amino sequence of any one of SEQ ID NOs:1-21 with four or fewer amino acid substitutions.

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Hsap MALVLGSLLLLGLCGNSFSGGQPSSTDAPKAWNYELPATNYETQDSHKAGPIGILFELVH 60
Ptro MALVLGSLLLLGLCGNSFSGGQPSSTDAPKAWNYELPATNYETQDSHKAGPIGILFELVH 60
Mmul MALVLGSLLLLGLCGNSFSGGQPSSTDAPKAWNYELPATNYETQDSHTAGPIGILFELVH 60
Rnor MALVFSVLLLLGLCGKMASGGQPAFDNTPGALNYELPTTEYETQDTFNAGIIDPLYQMVH 60
Mmus MALVFSALLLLGLCGKISSEGQPAFHNTPGAMNYELPTTKYETQDTFNAGIVGPLYKMVH 60
Cfam MALLLGFLLLLELCWDTSALGPLSSTKGSDGLEFELPATNYETKDSNQAGPISVLFQIVQ 60
Btau MALLLGFLLLLGLCEDTLSEELSSSGYRPDGLEFQLPPTSYQTSDSYDPGLAGFFFQIVR 60
     ***::. **** ** .  :    :    . . :::**.*.*:*.*:  .*  . ::::*:

Hsap IFLYVVQPRDFPEDTLRKFLQ-KAYESKIDYDKPETVILGLKIVYYEAGIILCCVLGLLF 119
Ptro IFLYVVQPRDFPEDTLRKVIQ-KAYESKIDYDKPETVILGLKIVYYEAGIILCCVLGLLF 119
Mmul IFLYVVQPRDFPEDTLRKVIQ-KARESKIDYDKPETLILGLKIIYYEAGIILCSVLGLLF 119
Rnor IFLNVVQPNDFPQDLVKKLIQ-KRFDISVDTKE---------VAIYEIGVLICVILGLLF 110
Mmus IFLSVVQPNDFPLDLIKKLIQNKKFDISVDSKEPEIIVLALKIALYEIGVLICAILGLLF 120
Cfam VFLQVVQPHPFPEDILRKILQ-KKFDFSTDYDK---------IIYYEIGIIICAVLGLLF 110
Btau FFVQIVQPNAFPEDILRKIIQ-KKFDLSKEYDKPENVVLTLKIIYYEIGIIICAALGLLF 119
     .*: :***. ** * ::*.:* *  : . : .:         :  ** *:::*  *****

Hsap IILMPLVGYFFCMCRCCNKCGGEMHQRQKENGPFLRKCFAISLLVICIIISIGIFYGFVA 179
Ptro IILMPLVGYFFCMCRCCNKCGGEMHQRQKENGPFLRKCFAISLLVICIIISIGIFYGFVA 179
Mmul IILMPLVGYFFCMCRCCNKCGGEMHQRQKENGLFLRKCFAISLLVICIIISIGIFCGFVA 179
Rnor IFLMPLVGFFFCMCRCCNKCGGEMHQRQKQNESCRRKCLAISLLLICLLMSLGIAFGFVA 170
Mmus IILMPLVGCFFCMCRCCNKCGGEMHQRQKQNAPCRRKCLGLSLLVICLLMSLGIIYGFVA 180
Cfam VILMPLVGFCFGLCRCCNKCGGEMHQRQKKNGAFLRKYFTVSLLVICIFISVGIIYGFVA 170
Btau VILMPLVGFFFCLCRCCNKCGGEMHQRQKKSGPFLKKYFTISLLVICVFISIGIIYGFAA 179
     ::******  * :**************:.    :* : :***:**:::*:**  **.*

Hsap NHQVRTRIKRSRKLADSNFKDLRTLLNETPEQIKYILAQYNTTKDKAFTDLNSINSVLGG 239
Ptro NHQVRTRIKRSRKLADSNFKDLRTLLNETPEQIKYILAQYNTTKDKAFTDLNSINSVLGG 239
Mmul NHQVRTRIKRSRKLADSNFKDLRTLLNETPEQIKYILAQYNTTKDKAFSDLNSINSVLGG 239
Rnor NQQTRTRIQRTQKLAESNYRDLRALLTEAPKQIDYILGQYNTTKNKAFSDLDSIDSVLGG 230
Mmus NQQTRTRIKGTQKLAKSNFRDFQTLLTETPKQIDYVVEQYTNTKNKAFSDLDGIGSVLGG 240
Cfam NHHLRTRIEKTRKLAESNLKDLRTLLIGTPAQINYVLSQYASTKEKAFSDLDNIKSLLGG 230
Btau NHYMRTNVEETRKLSESNLNDLRTLLNVVPGQIDYILDQFTLPKEKAFDDLDNINLLVGG 239
     *:  **.:: ::**:.** .*:::**  .* **.*:: *:  .*:*** **:.*  ::**

Hsap GILDRLRPNIIPVLDEIKSMATA-------------------------IKETKEALENMN 274
Ptro GILDRLRPNIIPVLDEIKSMATA-------------------------IKETKEALENMN 274
Mmul GILDRLRPNIIPVLDEIKSMATA-------------------------IKETKEALENMN 274
Rnor RIKGQLKPKVTPVLEEIKAMATA-------------------------IRQTKDALQNMS 265
Mmus RIKDQLKPKVTPVLEEIKAMATA-------------------------IKQTKDALQNMS 275
Cfam GIHDQLRPKVIPVLDDIKAMAEA-------------------------IKETREALLNVN 265
Btau SIYERLKPKVLPVLKDIKDLAEDGKGFFPPLVSPVGASVLKQAVFLTDMKTNRDTLVRMN 299
      *  :*:*:: ***.:** :*                            :: .:::* .:.
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FIG. 1A

WO 2010/129895 A2

GM, KE, LR, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

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CD133 EPITOPES

CLAIM OF PRIORITY

This application claims priority to U.S. Patent Application Serial No. 61/176,302, filed on May 7, 2009, the entire contents of which are hereby incorporated by reference.

TECHNICAL FIELD

This invention relates to methods and compositions for the treatment of cancers.

BACKGROUND

The cell surface marker CD133 (Prominin 1) is expressed by neural stem cells and has been used to select for brain cancer stem cells. In addition, CD133 positive cells are highly enriched for cancer stem cells in colon cancer, hepatocellular carcinoma, prostate cancer, multiple myeloma, and melanoma.

SUMMARY

This invention is based, in part, on the discovery of peptides of human CD133 that bind to human leukocyte antigens (HLA) and can stimulate immune responses. These peptides can be used in immunotherapy of cancers. Accordingly, compositions for cancer immunotherapy and methods for inducing immune responses in cancer patients against tumor antigens are provided herein.

In one aspect, the invention features an immunogen that includes an isolated peptide that includes the amino sequence of any of SEQ ID NOs:1-21 with four or fewer (e.g., three or fewer, two or fewer, one, or zero) amino acid substitutions (e.g., conservative substitutions). In some embodiments, the immunogen is 800 amino acid residues or fewer (e.g., 700 amino acid residues or fewer, 600 amino acid residues or fewer, 500 amino acid residues or fewer, 400 amino acid residues or fewer, 300 amino acid residues or fewer, 200 amino acid residues or fewer, 150 amino acid residues or fewer, 100 amino acid residues or fewer, 80 amino acid residues or fewer, 60 amino acid residues or fewer, 50 amino acid residues or fewer, 40 amino acid residues or fewer, 30 amino acid residues or fewer, 20 amino acid residues or fewer, 15 amino acid residues or fewer, 14 amino acid residues or fewer, 13 amino acid residues or fewer, 12 amino acid

residues or fewer, 11 amino acid residues or fewer, 10 amino acid residues or fewer, or 9 amino acid residues). In some embodiments, the immunogen comprises no more than 800 consecutive amino acid residues (e.g., no more than 700 amino acid residues, no more than 600 amino acid residues, no more than 500 amino acid residues, no more than 400 amino acid residues, no more than 300 amino acid residues, no more than 200 amino acid residues, no more than 150 amino acid residues, no more than 100 amino acid residues, no more than 80 amino acid residues, no more than 60 amino acid residues, no more than 50 amino acid residues, no more than 40 amino acid residues, no more than 30 amino acid residues, no more than 20 amino acid residues, no more than 15 amino acid residues, no more than 14 amino acid residues, no more than 13 amino acid residues, no more than 12 amino acid residues, no more than 11 amino acid residues, no more than 10 amino acid residues, or no more than or 9 amino acid residues) of SEQ ID NO:26. In some embodiments, the immunogen includes a superagonist variant of any of SEQ ID NOs:1-21. In some embodiments, the immunogen does not include the sequence FLLPALIFAV (SEQ ID NO:27).

In another aspect, the invention features compositions that include an immunogen described herein linked to an immunogenic carrier, e.g., a serum albumin, tetanus toxoid, keyhole limpet hemocyanin, dextran, an agonist of a Toll-like receptor (TLR), or a recombinant virus particle.

In another aspect, the invention features polynucleotides that include a nucleic acid sequence encoding an immunogen described herein. The polynucleotides can include an expression vector, e.g., a plasmid or a nonreplicative viral vector (e.g., vaccinia, fowlpox, Venezuelan equine encephalitis virus, adeno-associated virus, and adenovirus). In some embodiments the expression vector is a virus, e.g., an RNA or DNA virus.

In another aspect, the invention features compositions (e.g., pharmaceutical or vaccine compositions) that include an immunogen or polynucleotide described herein. The compositions can further include an adjuvant (e.g., complete Freund's adjuvant, incomplete Freund's adjuvant, Montanide ISA-51, LAG-3, aluminum phosphate, aluminum hydroxide, alum, or saponin), a cytokine (e.g., Interleukin-1 (IL-1), IL-2, IL-7, IL- 12, IL-13, IL-15, tumor necrosis factor (TNF), stem cell factor (SCF), or granulocyte

monocyte colony stimulating factor (GM-CSF)), and/or an agonist of a Toll-like receptor (TLR) (e.g., an agonist of TLR-3, TLR-4, TLR-7, or TLR-9). The compositions can include a vehicle, e.g., a liposome (e.g., an emulsion, a foam, a micel, an insoluble monolayer, a liquid crystal, a phospholipid dispersion, or a lamellar layer), an immuno stimulating complex (ISCOM), or a slow-releasing particle.

In a further aspect, the invention features methods of immunization that include administering to a subject an immunogen, polynucleotide, or composition described herein in an amount effective to stimulate an immune response (e.g., a therapeutic or prophylactic immune response). The invention also features the use of an immunogen, polynucleotide, or composition described herein in the preparation of a medicament for stimulating an immune response. The invention also features the use of an immunogen, polynucleotide, or composition described herein to stimulate an immune response.

In another aspect, the invention features methods for treating a subject with a cancer (e.g., a cancer characterized by tumor cells expressing a class I MHC molecule). The methods include administering to the subject an immunogen, polynucleotide, or composition described herein in an amount effective to induce a CTL response to the tumor cells. The invention also features the use of an immunogen, polynucleotide, or composition described herein in the preparation of a medicament for treating a subject with a cancer (e.g., a cancer characterized by tumor cells expressing a class I MHC molecule). The invention also features the use of an immunogen, polynucleotide, or composition described herein for treating a subject with a cancer (e.g., a cancer characterized by tumor cells expressing a class I MHC molecule).

In a further aspect, the invention features methods for treating a subject with a cancer characterized by tumor cells expressing HLA-A1, HLA-A2, or HLA-A3. The methods include administering to the subject induced cytotoxic T lymphocyte (CTLs) in an amount sufficient to destroy the tumor cells through direct lysis or to effect the destruction of the tumor cells indirectly through the elaboration of cytokines, wherein the CTLs are induced by a process that includes inducing a CTL in vitro that is specific for the tumor cells by contacting a precursor CTL with an immunogen described herein under conditions that generate a CTL response to the tumor cells. The invention also features the use of an immunogen, polynucleotide, or composition described herein in the

preparation of a medicament for treating a subject with a cancer characterized by tumor cells expressing HLA-A1, HLA-A2, or HLA-A3. The invention also features the use of an immunogen, polynucleotide, or composition described herein for treating a subject with a cancer characterized by tumor cells expressing HLA-A1, HLA-A2, or HLA-A3.

In another aspect, the invention features methods for treating a subject with a cancer characterized by tumor cells expressing any class I MHC molecule. The methods include administering to the subject induced cytotoxic T lymphocyte (CTLs) in an amount sufficient to destroy the tumor cells through direct lysis or to effect the destruction of the tumor cells indirectly through the elaboration of cytokines, said CTLs are induced by a process comprising inducing a CTL in vitro that is specific for said tumor cells by contacting a precursor CTL with an immunogen described herein under conditions that generate a CTL response to the tumor cells.

In a further aspect, the invention features methods for inducing a cytotoxic T lymphocyte (CTL) in vitro that is specific for a tumor cell expressing HLA-A1, HLA-A2, or HLA-A3. The methods include contacting a precursor CTL with an immunogen described herein under conditions that generate a CTL response to the tumor cells.

In another aspect, the invention features methods for inducing a cytotoxic T lymphocyte (CTL) response in vitro that is specific for a tumor cell expressing HLA-A1, HLA-A2, or HLA-A3. The methods include contacting a precursor CTL with a cell that includes a polynucleotide having a nucleic acid sequence encoding at least one polypeptide that includes an immunogen described herein.

In a further aspect, the invention features methods for treating a subject with a cancer characterized by tumor cells expressing HLA-A1, HLA-A2, or HLA-A3. The methods include administering CTLs induced by a method described herein in an amount effective to destroy the tumor cells through direct lysis or to effect the destruction of the tumor cells indirectly through the elaboration of cytokines.

The invention also features methods for treating a cancer in a patient that include administering to the patient a composition comprising antigen-presenting cells (e.g., dendritic cells), wherein the antigen presenting cells present on their surface a peptide epitope comprising the amino sequence of any of SEQ ID NOs:1-21 with four or fewer (e.g., three or fewer, two or fewer, one, or zero) amino acid substitutions (e.g.,

conservative substitutions) or a superagonist variant of any of SEQ ID NOs:1-21. In some embodiments, the antigen presenting cells (e.g., dendritic cells) acquire the peptide epitopes in vitro by exposure to synthetic peptides having the peptide epitopes. The invention also features the use of antigen presenting cells that present on their surface an immunogen described herein in the preparation of a medicament for treating a subject with cancer. The invention also features the use of antigen presenting cells that present on their surface an immunogen described herein for treating a subject with cancer.

In a further aspect, the invention features methods for preparing a cell vaccine for treating a cancer. The methods include: obtaining bone marrow derived mononuclear cells from a patient, culturing the mononuclear cells in vitro under conditions in which mononuclear cells become adherent to a culture vessel; selecting a subset of the mononuclear cells comprising adherent cells; culturing the adherent cells in the presence of one or more cytokines under conditions in which the cells differentiate into antigen presenting cells; and culturing the antigen presenting cells in the presence of an immunogen described herein under conditions in which the cells present the peptides on major histocompatibility class I molecules, thereby preparing a cell vaccine.

In any of the above aspects, a cancer or tumor may include one or more cells that express CD133.

In another aspect, the invention features kits that include one or more immunogens, polynucleotides, and/or compositions described herein.

A "superagonist" or "superantigen" peptide is a peptide that includes one or more mutations (e.g., one, two, or three amino acid changes, relative to a native (wild type) sequence) and that elicits an antigen-specific immunological response that is more potent than a response elicited against a peptide having a native sequence. For example, a superagonist peptide stimulates higher levels of IFN-γ release by antigen-specific T cells, as compared to T cells stimulated with the native peptide. The increase in levels of IFN-γ release stimulated by a superagonist peptide is at least higher than levels stimulated by a native peptide by a statistically significant amount. In some embodiments, a superagonist stimulates IFN-γ levels that are at least 5%, 10%, 25%, 50%, 100%, 200%, or 500% higher than elicited by the native peptide.

Unless defined otherwise, technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Singleton et al., Dictionary of Microbiology and Molecular Biology 3rd ed., J. Wiley & Sons (New York, NY 2001); March, Advanced Organic Chemistry Reactions, Mechanisms and Structure 5th ed., J. Wiley & Sons (New York, NY 2001); Sambrook and Russel, Molecular Cloning: A Laboratory Manual 3rd ed., Cold Spring Harbor Laboratory Press (Cold Spring Harbor, NY 2001); and Lutz et al., Handbook of Dendritic Cells: Biology, Diseases and Therapies, J. Wiley & Sons (New York, NY 2006), provide one skilled in the art with a general guide to many of the terms used in the present application. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

The details of one or more embodiments of the invention are set forth in the description below. Other features, objects, and advantages of the invention will be apparent from the description and from the claims.

DESCRIPTION OF THE DRAWINGS

Figs. 1A-C depict a multiple alignment of human (Hsap, SEQ ID NO:26), chimpanzee (Ptro, SEQ ID NO:30), Rhesus macaque (Mmul, SEQ ID NO:31), rat (Rnor, SEQ ID NO:32), mouse (Mmus, SEQ ID NO:33), dog (Cfam, SEQ ID NO:34), and cattle (Btau, SEQ ID NO:35) CD133 proteins. The multiple alignment was prepared using ClustalW2 (Larkin et al., 2007, Bioinformatics, 23:2947-48). "*", residues are identical in all sequences; ":", conserved substitutions; ".", semi-conserved substitutions.

DETAILED DESCRIPTION

The present invention relates to immunogens and immunogenic compositions, and methods of use thereof, for the prevention, treatment, and/or diagnosis of cancers.

Described herein are immunogens that include proteins or polypeptides whose amino acid sequences include one or more epitopic oligopeptides. In addition, the invention further relates to polynucleotides that can be used to stimulate a CTL response against cancers.

Described herein are specific oligopeptide sequences with amino acid sequences shown in SEQ ID NOs:1-21, which represent epitopic peptides (i.e., immunogenic oligopeptide sequences) of at least about 9-10 amino acids in length.

CD133 is present in several human cancers (Mizrak et al., 2008, J. Pathol., 214:3-9; Neuzil et al., 2007, Biochem. Biophys. Res. Commun., 355:855-859), including brain cancer, colon cancer, hepatocellular carcinoma, prostate cancer, multiple myeloma, and melanoma.

An exemplary human CD133 sequence has the following amino acid sequence (SEQ ID NO:26).

MALVLGSLLLLGLCGNSFSGGQPSSTDAPKAWNYELPATNYETQDSHKA
GPIGILFELVHIFLYVVQPRDFPEDTLRKFLQKAYESKIDYDKPETVIL
GLKIVYYEAGIILCCVLGLLFIILMPLVGYFFCMCRCCNKCGGEMHQRQ
KENGPFLRKCFAISLLVICIIISIGIFYGFVANHQVRTRIKRSRKLADS
NFKDLRTLLNETPEQIKYILAQYNTTKDKAFTDLNSINSVLGGGILDRL
RPNIIPVLDEIKSMATAIKETKEALENMNSTLKSLHQQSTQLSSSLTSV
KTSLRSSLNDPLCLVHPSSETCNSIRLSLSQLNSNPELRQLPPVDAELD
NVNNVLRTDLDGLVQQGYQSLNDIPDRVQRQTTTVVAGIKRVLNSIGSD
IDNVTQRLPIQDILSAFSVYVNNTESYIHRNLPTLEEYDSYWWLGGLVI
CSLLTLIVIFYYLGLLCGVCGYDRHATPTTRGCVSNTGGVFLMVGVGLS
FLFCWILMIIVVLTFVFGANVEKLICEPYTSKELFRVLDTPYLLNEDWE
YYLSGKLFNKSKMKLTFEQVYSDCKKNRGTYGTLHLQNSFNISEHLNIN
EHTGSISSELESLKVNLNIFLLGAAGRKNLQDFAACGIDRMNYDSYLAQ
TGKSPAGVNLLSFAYDLEAKANSLPPGNLRNSLKRDAQTIKTIHQQRVL
PIEQSLSTLYQSVKILQRTGNGLLERVTRILASLDFAQNFITNNTSSVI
IEETKKYGRTIIGYFEHYLQWIEFSISEKVASCKPVATALDTAVDVFLC

SYIIDPLNLFWFGIGKATVFLLPALIFAVKLAKYYRRMDSEDVYDDVET
IPMKNMENGNNGYHKDHVYGIHNPVMTSPSQH (SEQ ID NO:26)

The polypeptides forming the immunogens described herein have amino acid sequences that include SEQ ID NOs:1-21 and variants thereof with four or fewer (e.g., three or fewer, two or fewer, one, or zero) amino acid substitutions (e.g., conservative substitutions).

Such polypeptides can be of any desired length so long as they have immunogenic activity in that they are able, under a given set of conditions, to elicit in vitro or in vivo the activation of cytotoxic T lymphocytes (CTLs) (i.e., a CTL response) against a presentation of CD133 in vitro or in vivo by an antigen presenting cell (APC). Exemplary polypeptides include those of 800 amino acid residues or fewer (e.g., 700 amino acid residues or fewer, 600 amino acid residues or fewer, 500 amino acid residues or fewer, 400 amino acid residues or fewer, 300 amino acid residues or fewer, 200 amino acid residues or fewer, 150 amino acid residues or fewer, 100 amino acid residues or fewer, 80 amino acid residues or fewer, 60 amino acid residues or fewer, 50 amino acid residues or fewer, 40 amino acid residues or fewer, 30 amino acid residues or fewer, 20 amino acid residues or fewer, 15 amino acid residues or fewer, 14 amino acid residues or fewer, 13 amino acid residues or fewer, 12 amino acid residues or fewer, 11 amino acid residues or fewer, 10 amino acid residues or fewer, or 9 amino acid residues). The polypeptides forming the immunogens described herein can be naturally occurring or can be synthesized chemically. The polypeptides can include at least one of SEQ ID NOs:1-21.

In some embodiments, an immunogen described herein can a variant sequence such as the counterpart of any of SEQ ID NOs:1-21 from the CD133 protein of an animal species (e.g., chimpanzee, Rhesus macaque, rat, mouse, dog, or cattle). A counterpart peptide can be identified by aligning the human and animal CD133 proteins (e.g., as shown in Figs. 1A-C) and selecting the sequence from the animal protein that aligns with the portion of the human sequence corresponding to the peptide of interest. For example, SEQ ID NO:11 and its animal counterparts are shown in underscore in Fig. 1C. In some

instances, the counterpart sequence immunogen may have more than four amino acid differences as compared to the human sequence.

Oligopeptides as disclosed herein may themselves be prepared by methods well known to those skilled in the art. See, e.g., Grant, G. A., Synthetic Peptides: A User's Guide, 1992, W. H. Freeman and Company, New York; Coligan, J. E. et al, Current Protocols in Protein Science, 1999, John Wiley & Sons, Inc., New York.

Besides the sequences of SEQ ID NOs:1-21, the proteins and polypeptides forming the immunogens described herein can also include one or more other immunogenic amino acid stretches known to be associated with cancers, and which may stimulate a CTL response whereby the immunogenic peptides associate with HLA-A1, HLA-A2, HLA-A3, HLA-A1/A11, HLA supertypes, or any class I MHC (i.e., MHC-1) molecule.

The oligopeptides and polypeptides described herein can be derived by fractionation of naturally occurring proteins by methods such as protease treatment, or they can be produced by recombinant or synthetic methodologies that are well known and clear to the skilled artisan. See, e.g., Ausubel, F. M. et al, Current Protocols in Molecular Biology, 1999, John Wiley & Sons, Inc., New York; Coligan, J. E. et al, Current Protocols in Protein Science, 1999, John Wiley & Sons, Inc., New York; Molecular Cloning: A Laboratory Manual, 1989, Cold Spring Harbor Laboratory Press, Cold Spring Harbor. The polypeptide can include a recombinant or synthetic polypeptide that includes at least one of SEQ ID NOs:1-21, which sequences can also be present in multiple copies. Thus, oligopeptides and polypeptides disclosed herein can have one, two, three, or more such immunogenic peptides within the amino acid sequence of said oligopeptides and polypeptides, and said immunogenic peptides, or epitopes, can be the same or can be different, or can have any number of such sequences, wherein some of them are identical to each other in amino acid sequence while others within the same polypeptide sequence are different from each other and said epitopic sequences can occur in any order within said immunogenic polypeptide sequence. The location of such sequences within the sequence of a polypeptide forming an immunogen described herein can affect relative immunogenic activity. In addition, immunogens described herein can include more than one protein comprising the amino acid sequences disclosed herein.

Such polypeptides can be part of a single composition or can themselves be covalently or non-covalently linked to each other.

The immunogenic peptides described herein can also be linked directly to, or through a spacer or linker to: an immunogenic carrier such as serum albumin, tetanus toxoid, keyhole limpet hemocyanin, dextran, or a recombinant virus particle; a Toll-like receptor (TLR) agonist; an immunogenic peptide known to stimulate a T helper cell type immune response; a cytokine such as interferon gamma or GM-CSF; a targeting agent such as an antibody or receptor ligand; a stabilizing agent such as a lipid; or a conjugate of a plurality of epitopes to a branched lysine core structure, such as the so-called "multiple antigenic peptide" described in Posneft et al., 1988, J. Biol. Chem., 263:1719-1725; a compound such as polyethylene glycol to increase the half life of the peptide; or additional amino acids such as a leader or secretory sequence, or a sequence employed for the purification of the mature sequence. Spacers and linkers typically include relatively small, neutral molecules, such as amino acids and which are substantially uncharged under physiological conditions. Such spacers are typically selected from the group of nonpolar or neutral polar amino acids, such as glycine, alanine, serine and other similar amino acids. Such optional spacers or linkers need not include the same residues and thus can be either homo- or hetero-oligomers. When present, such linkers will commonly be of length at least one or two, commonly 3, 4, 5, 6, and possibly as much as 10 or even up to 20 residues (in the case of amino acids). In addition, such linkers need not be composed of amino acids but any oligomeric structures will do as well so long as they provide the correct spacing so as to optimize the desired level of immunogenic activity of the immunogens described herein. The immunogen can therefore take any form that is capable of eliciting a CTL response.

In addition, the immunogenic peptides described herein can be part of an immunogenic structure via attachments other than conventional peptide bonds. Thus, any manner of attaching the peptides to an immunogen described herein, such as an immunogenic polypeptide, could provide an immunogenic structure. Thus, immunogens, such as proteins, oligopeptides and polypeptides, are structures that contain the peptides disclosed, but such immunogenic peptides may not necessarily be attached thereto by the conventional means of using ordinary peptide bounds. The immunogens described herein

simply contain such peptides as part of their makeup, but how such peptides are to be combined to form the final immunogen is left to the talent and imagination of the user and is in no way restricted or limited by the disclosure contained herein.

It should be appreciated that an immunogen described herein can consist only of a peptide of SEQ ID NOs:1-21 (or a variant thereof), or include a peptide of SEQ ID NOs:1-21 (or a variant thereof), or include a plurality of peptides selected from SEQ ID NOs:1-21 (or one or more variants thereof), or include a polypeptide that itself includes one or more of the epitopic peptides of SEQ ID NOs:1-21 (or one or more variants thereof). In some embodiments, an immunogen, composition, or kit described herein can further include or exclude a polypeptide, epitope, or other antigenic composition described in US 2007/0020297; US 2008/0206296; US 2008/0311142; or WO 2010/028066, all of which are incorporated by reference herein.

Modified Peptides

The peptides that are naturally processed and bound to a class I MHC molecule, and which are recognized by a tumor-specific CTL, are not necessarily the optimal peptides for stimulating a CTL response. See, for example, Parkhurst et al., 1996, J. Immunol., 157:2539-48; Rosenberg et al., 1998, Nat. Med., 4:321-32. Thus, there can be utility in modifying a peptide, such that it more readily or effectively induces a CTL response. Typically, peptides can be modified at two types of positions. The peptides can be modified at amino acid residues that are predicted to interact with the class I MHC molecule, in which case the goal is to create a peptide that has a higher affinity for the class I MHC molecule than does the original peptide. The peptides can also be modified at amino acid residues that are predicted to interact with the T cell receptor on the CTL, in which case the goal is to create a peptide that has a higher affinity for the T cell receptor than does the original peptide. Both of these types of modifications can result in a variant peptide that is related to an original peptide, but which is better able to induce a CTL response than is the original peptide. As used herein, the term "original peptide" means an oligopeptide with the amino acid sequence selected from SEQ ID NOs:1-21.

The original peptides disclosed herein can be modified by the substitution of one or more residues at different, possibly selective, sites within the peptide chain. Such

substitutions can be of a conservative nature, for example, where one amino acid is replaced by an amino acid of similar structure and characteristics, such as where a hydrophobic amino acid is replaced by another hydrophobic amino acid. Even more conservative would be replacement of amino acids of the same or similar size and chemical nature, such as where leucine is replaced by isoleucine. In studies of sequence variations in families of naturally occurring homologous proteins, certain amino acid substitutions are more often tolerated than others, and these often show correlation with similarities in size, charge, polarity, and hydrophobicity between the original amino acid and its replacement, and such is the basis for defining "conservative substitutions."

Conservative substitutions are defined herein as exchanges within one of the following five groups: Group 1--small aliphatic, nonpolar or slightly polar residues (Ala, Ser, Thr, Pro, Gly); Group 2--polar, negatively charged residues and their amides (Asp, Asn, Glu, Gln); Group 3--polar, positively charged residues (His, Arg, Lys); Group 4--large, aliphatic, nonpolar residues (Met, Leu, Ile, Val, Cys); and Group 4--large, aromatic residues (Phe, Tyr, Trp).

Less conservative substitutions might involve the replacement of one amino acid by another that has similar characteristics, but is somewhat different in size, such as replacement of an alanine by an isoleucine residue. Highly nonconservative replacements might involve substituting an acidic amino acid for one that is polar, or even for one that is basic in character, or vice versa.

Such substitutions can also involve structures other than the common L-amino acids. Thus, D-amino acids might be substituted for the L-amino acids commonly found in the antigenic peptides described herein and yet still be encompassed by the present disclosure. In addition, amino acids possessing non-standard R groups (i.e., R groups other than those found in the 20 common amino acids of natural proteins) can also be used for substitution purposes to produce immunogens and immunogenic polypeptides.

Based on cytotoxicity assays, a substituted epitopic peptide is considered substantially identical to the reference peptide if it has at least 10% of the antigenic activity of the reference peptide as defined by the ability of the substituted peptide to reconstitute the epitope recognized by a CTL in comparison to the reference peptide. Thus, when comparing the lytic activity in the linear portion of the effector:target curves

with equimolar concentrations of the reference and substituted peptides, the observed percent specific killing of the target cells incubated with the substituted peptide should be equal to that of the reference peptide at an effector:target ratio that is no greater than 10-fold above the reference peptide effector:target ratio at which the comparison is being made.

Thus, the epitopes described herein can be identical to naturally occurring tumor-associated or tumor-specific epitopes or can include epitopes that differ by no more than 4 residues from the reference peptide, as long as they have substantially identical antigenic activity.

Preparation of Immunogenic Peptides and Structures

The immunogenic peptides and polypeptides described herein can be prepared synthetically, by recombinant DNA technology, or they can be isolated from natural sources such as tumor cells expressing the original protein product.

The polypeptides and oligopeptides disclosed herein can be synthesized in solution or on a solid support in accordance with conventional techniques. Various automated peptide synthesizers are commercially available and can be used in accordance with known protocols. See, for example, Grant, G. A., Synthetic Peptides: A User's Guide, 1992, W. H. Freeman and Company, New York; Coligan, J. E. et al, Current Protocols in Protein Science, 1999, John Wiley & Sons, Inc., New York. Fragments of polypeptides described herein can also be synthesized as intermediates in the synthesis of a larger polypeptide.

Recombinant DNA technology can be employed wherein a nucleotide sequence that encodes an immunogenic peptide or polypeptide of interest is inserted into an expression vector, transformed or transfected into an appropriate host cell, and cultivated under conditions suitable for expression. These procedures are well known in the art to the skilled artisan, as described in, e.g., Coligan, J. E. et al, Current Protocols in Immunology, 2006, John Wiley & Sons, Inc., New York; Ausubel, F. M. et al, Current Protocols in Molecular Biology, 1999, John Wiley & Sons, Inc., New York; Molecular Cloning: A Laboratory Manual, 1989, Cold Spring Harbor Laboratory Press, Cold Spring

Harbor. Thus, recombinantly produced peptides or polypeptides can be used as the immunogens described herein.

The coding sequences for peptides of the length contemplated herein can also be synthesized on commercially available automated DNA synthesizers using protocols that are well know in the art. See for example, Grant, G. A., Synthetic Peptides: A User's Guide, 1992, W. H. Freeman and Company, New York; Coligan, J. E. et al, Current Protocols in Protein Science, 1999, John Wiley & Sons, Inc., New York. The coding sequences can also be modified such that a peptide or polypeptide will be produced that incorporates a desired amino acid substitution. The coding sequence can be provided with appropriate linkers, be ligated into suitable expression vectors that are commonly available in the art, and the resulting DNA or RNA molecule can be transformed or transfected into suitable hosts to produce the desired fusion protein. A number of such vectors and suitable host systems are available, and their selection is left to the skilled artisan. For expression of the fusion proteins, the coding sequence will be provided with operably linked start and stop codons, promoter and terminator regions, and a replication system to provide an expression vector for expression in the desired host cell. For example, promoter sequences compatible with bacterial hosts are provided in plasmids containing convenient restriction sites for insertion of the desired coding sequence. The resulting expression vectors are transformed into suitable bacterial hosts. Yeast, insect, and mammalian host cells can also be used, employing suitable vectors and control sequences.

Host cells can be genetically engineered (e.g., transduced, transformed, or transfected) with the vectors described herein which can be, for example, a cloning vector or an expression vector. The vector can be, for example, in the form of a plasmid, a viral particle, a phage, etc. The engineered host cells can be cultured in conventional nutrient media modified as appropriate for activating promoters, selecting transformants or amplifying the genes. The culture conditions, such as temperature, pH and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

The present invention also includes recombinant constructs comprising one or more of the sequences as broadly described above. The constructs include a vector, such

as a plasmid or viral vector, into which a sequence described herein has been inserted, in a forward or reverse orientation. In a preferred aspect of this embodiment, the construct further includes regulatory sequences, including, for example, a promoter, operably linked to the sequence. Large numbers of suitable vectors and promoters are known to those of skill in the art, and are commercially available.

Host Cells

In a further embodiment, the present invention relates to host cells containing the above-described constructs. The host cell can be a higher eukaryotic cell, such as a mammalian cell, or a lower eukaryotic cell, such as a yeast cell, or the host cell can be a prokaryotic cell, such as a bacterial cell. Introduction of the construct into the host cell can be effected by calcium phosphate transfection, DEAE-Dextran mediated transfection, or electroporation. See, e.g., Ausubel, F. M. et al, Current Protocols in Molecular Biology, 1999, John Wiley & Sons, Inc., New York; Molecular Cloning: A Laboratory Manual, 1989, Cold Spring Harbor Laboratory Press, Cold Spring Harbor. Such cells can routinely be utilized for assaying CTL activity by having said genetically engineered, or recombinant, host cells express the immunogenic peptides described herein.

Various mammalian cell culture systems can also be employed to express recombinant protein. Examples of mammalian expression systems include the COS-7 lines of monkey kidney fibroblasts, described by Gluzman, 1981, Cell, 23:175, and other cell lines capable of expressing a compatible vector, for example, the C127, 3T3, CHO, HeLa and BHK cell lines. Mammalian expression vectors will include an origin of replication, a suitable promoter and enhancer, and also any necessary ribosome binding sites, polyadenylation site, splice donor and acceptor sites, transcriptional termination sequences, and 5' flanking non-transcribed sequences. DNA sequences derived from the SV40 splice, and polyadenylation sites can be used to provide the required nontranscribed genetic elements.

The polypeptides can be recovered and purified from recombinant cell cultures by methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography

and lectin chromatography. Protein refolding steps can be used, as necessary, in completing configuration of the mature peptides and proteins. High performance liquid chromatography (HPLC) can be employed for final purification steps.

Antigen-Presenting Cells

Antigen presenting cells that are to be used to stimulate a CTL response are typically incubated with a peptide of an optimal length, for example a nonapeptide, that allows for direct binding of the peptide to the class I MHC molecule without additional processing. Larger oligopeptides and polypeptides are generally ineffective in binding to class I MHC molecules as they are not efficiently processed into an appropriately sized peptide in the extracellular milieu. A variety of approaches are known in the art, however, that allow oligopeptides and polypeptides to be exogenously acquired by a cell, which then allows for their subsequent processing and presentation by a class I MHC molecule. Representative, but non-limiting examples of such approaches include electroporation of the molecules into the cell (Harding, 1992, Eur. J. Immunol., 22:1865-69), encapsulation of the molecules in liposomes that are fused to the cells of interest (Reddy et al., 1991, J. Immunol. Methods, 141:157-163), or osmotic shock in which the molecules are taken up via pinocytosis (Moore et al., 1988, Cell, 54:777-785). Thus, oligopeptides and polypeptides that include one or more of the peptides described herein can be provided to antigen presenting cells in such a fashion that they are delivered to the cytoplasm of the cell, and are subsequently processed to allow presentation of the peptides.

Antigen presenting cells suitable for stimulating an in vitro CTL response that is specific for one or more of the peptides described herein can also be prepared by introducing polynucleotide vectors encoding the sequences into the cells. These polynucleotides can be designed such that they express only a single peptide, multiple peptides, or even a plurality of peptides. A variety of approaches are known in the art that allow polynucleotides to be introduced and expressed in a cell, thus providing one or more peptides described herein to the class I MHC molecule binding pathway. Representative, but non-limiting examples of such approaches include the introduction of plasmid DNA through particle-mediated gene transfer or electroporation (Tuting et al.,

1998, J. Immunol., 160:1139-47), or the transduction of cells with an adenovirus expressing the polynucleotide of interest (Perez-Diez et al., 1998, Cancer Res., 58:5305-09). Thus, oligonucleotides that code for one or more of the peptides described herein can be provided to antigen presenting cells in such a fashion that the peptides associate with class I MHC molecules and are presented on the surface of the antigen presenting cell, and consequently are available to stimulate a CTL response.

In certain embodiments, the methods described herein include a method for inducing a CTL response in vitro that is specific for a tumor cell expressing a molecule from A1, A2, or A3 supertypes (A11 is a member of the A3 supertype), whereby the method includes contacting a CTL precursor lymphocyte with an antigen presenting cell that has bound to an immunogen comprising one or more of the peptides disclosed herein.

In specific embodiments, the methods described herein include a method for inducing a CTL response in vitro that is specific for a tumor cell expressing a molecule from A1, A2, or A3 supertypes, whereby the method includes contacting a CTL precursor lymphocyte with an antigen presenting cell that has exogenously acquired an immunogenic oligopeptide or polypeptide that includes one or more of the peptides disclosed according to the invention.

A yet additional embodiment described herein is directed to a process for inducing a CTL response in vitro that is specific for a tumor cell expressing a molecule from A1, A2, or A3 supertypes, comprising contacting a CTL precursor lymphocyte with an antigen presenting cell that is expressing a polynucleotide coding for a polypeptide described herein, and wherein said polynucleotide is operably linked to a promoter.

A variety of techniques exist for assaying the activity of CTL. These techniques include the labeling of target cells with radionuclides such as $Na_2{}^{51}CrO_4$ or ^{3}H-thymidine, and measuring the release or retention of the radionuclides from the target cells as an index of cell death. Such assays are well-known in the art. Alternatively, CTL are known to release a variety of cytokines when they are stimulated by an appropriate target cell, such as a tumor cell expressing the relevant class I MHC molecule and the corresponding peptide. Non-limiting examples of such cytokines include IFN-γ, TNF-α, and GM-CSF. Assays for these cytokines are well known in the art. Methodology for

measuring both target cell death and cytokine release as a measure of CTL reactivity are given in Coligan, J. E. et al. (Current Protocols in Immunology, 1999, John Wiley & Sons, Inc., New York).

After expansion of the antigen-specific CTLs, the latter can then be transferred back into the patient, where they will destroy their specific target cell. The utility of such adoptive transfer is demonstrated in North et al. (199, Infect. Immun., 67:2010-12) and Riddell et al. (1992, Science, 257:238-241). In determining the number of cells to reinfuse, the skilled physician will be guided by the total number of cells available, the activity of the CTL as measured in vitro, and the condition of the patient. Typically, about $1x10^6$ to about $1x10^{12}$ (e.g., about $1x10^8$ to about $1x10^{11}$ or about $1x10^9$ to about $1x10^{10}$) peptide-specific CTL are infused. Methods for reinfusing T cells into a patient are well known and exemplified in U.S. Pat. No. 4,844,893 to Honski, et al., and U.S. Pat. No. 4,690,915 to Rosenberg.

The peptide-specific CTL can be purified from the stimulator cells prior to infusion into the patient. For example, monoclonal antibodies directed toward the cell surface protein CD8, present on CTL, can be used in conjunction with a variety of isolation techniques such as antibody panning, flow cytometric sorting, and magnetic bead separation to purify the peptide-specific CTL away from any remaining non-peptide specific lymphocytes or from the stimulator cells. These methods are well known in the art. It should be appreciated that generation of peptide-specific CTL in this manner obviates the need for stimulating the CTL in the presence of tumor. Thus, there is no chance of inadvertently reintroducing tumor cells into the patient.

Thus, one embodiment of the present invention relates to a process for treating a subject who has cancer characterized by tumor cells expressing complexes of a molecule from A1, A2, or A3 supertypes, for example, HLA-A1, HLA-A2, HLA-A3, or HLAA11, whereby CTLs produced in vitro according to the methods described herein are administered in an amount sufficient to destroy the tumor cells through direct lysis or to effect the destruction of the tumor cells indirectly through the elaboration of cytokines.

Another embodiment of the present invention is directed to a process for treating a subject with cancer characterized by tumor cells expressing any class I MHC molecule and an epitope of SEQ ID NOs:1-21, whereby the CTLs are produced in vitro and are

specific for the epitope or original protein and are administered in an amount sufficient to destroy the tumor cells through direct lysis or to effect the destruction of the tumor cells indirectly through the elaboration of cytokines.

The ex vivo generated CTL can be used to identify and isolate the T cell receptor molecules specific for the peptide. The genes encoding the alpha and beta chains of the T cell receptor can be cloned into an expression vector system and transferred and expressed in naive T cells from peripheral blood, T cells from lymph nodes, or T lymphocyte progenitor cells from bone marrow. These T cells, which would then be expressing a peptide-specific T cell receptor, would then have anti-tumor reactivity and could be used in adoptive therapy of cancers.

Screening and Diagnostic Methods

In addition to their use for therapeutic or prophylactic purposes, the immunogenic peptides described herein are useful as screening and diagnostic agents. Thus, the immunogenic peptides described herein, together with modern techniques of gene screening, make it possible to screen patients for the presence of genes encoding such peptides on cells obtained by biopsy of tumors detected in such patients. For example, patients can be screened using nucleic acids or antibodies to detect the expression of CD133. The results of such screening can help determine the efficacy of proceeding with the regimen of treatment disclosed herein using the immunogens described herein.

Alternatively, the immunogenic peptides disclosed herein, as well as functionally similar homologs thereof, can be used to screen a sample for the presence of CTLs that specifically recognize the corresponding epitopes. The lymphocytes to be screened in this assay will normally be obtained from the peripheral blood, but lymphocytes can be obtained from other sources, including lymph nodes, spleen, tumors, and pleural fluid. The peptides described herein can then be used as a diagnostic tool to evaluate the efficacy of the immunotherapeutic treatments disclosed herein. Thus, the in vitro generation of CTL as described above would be used to determine if patients are likely to respond to the peptide in vivo. Similarly, the in vitro generation of CTL could be done with samples of lymphocytes obtained from the patient before and after treatment with the peptides. Successful generation of CTL in vivo should then be recognized by a

correspondingly easier ability to generate peptide-specific CTL in vitro from lymphocytes obtained following treatment in comparison to those obtained before treatment.

The oligopeptides described herein, such as SEQ ID NOs:1-21, can also be used to prepare multimers (e.g., dimers, tetramers, or pentamers), which can be used, e.g., in conjunction with flow cytometry, to quantitate the frequency of peptide-specific CTL that are present in a sample of lymphocytes from an individual. For example, class I MHC molecules comprising peptides of SEQ ID NOs:1-21, could be combined to form tetramers as exemplified in U.S. Pat. No. 5,635,363. The multimers (e.g., tetramers) can be used in monitoring the frequency of CTLs in the peripheral blood, lymph nodes, or tumor mass of an individual undergoing immunotherapy with the peptides, proteins, or polynucleotides described herein, and it would be expected that successful immunization would lead to an increase in the frequency of the peptide-specific CTL. A description of peptide tetramers and methods of using them can be found in Coligan et al, Current Protocols in Immunology, 2006, John Wiley & Sons, Inc., New York.

Methods of Therapy

A vaccine can include one or more of the polypeptides or fragments thereof described herein, or a composition, or pool, of immunogenic peptides disclosed herein. Two or more polypeptides and/or fragments thereof can be used as a physical mixture or as a fusion. The fusion fragment or fusion polypeptide can be produced, for example, by recombinant techniques or by the use of appropriate linkers for fusing previously prepared polypeptides or fragments.

The immunogenic molecules described herein, including vaccine compositions, can be utilized according to the methods described herein for purposes of inhibiting, suppressing, or treating diseases causing the expression of the immunogenic peptides disclosed herein, such as where the antigen is being expressed by tumor cells. As used in accordance with the present application, the term "inhibiting" relates to a process of prophylaxis in which an animal, especially a mammal, and most especially a human, is exposed to an immunogen described herein prior to the induction or onset of the disease process. This could be done where an individual has a genetic pedigree indicating a

predisposition toward occurrence of the disease condition to be prevented. For example, this might be true of an individual whose ancestors show a predisposition toward certain types of cancer. Alternatively, the immunogen could be administered to the general population as is frequently done for infectious diseases.

The term "suppression" is often used to describe a scenario wherein the disease process has already begun, but obvious symptoms of said condition have yet to be realized. Thus, the cells of an individual may have become cancerous, but no outside signs of the disease have yet been clinically recognized. The term prophylaxis is used herein to encompass both inhibition and suppression. Conversely, the term "treatment" is used herein to mean the clinical application of agents to combat an already existing condition whose clinical presentation has already been realized in a patient. This would typically occur where an individual has already been diagnosed as having a tumor.

As used herein, the term “cancer” refers to cells having the capacity for autonomous growth, i.e., an abnormal state or condition characterized by rapidly proliferating cell growth. Hyperproliferative and neoplastic disease states may be categorized as pathologic, i.e., characterizing or constituting a disease state, or may be categorized as non-pathologic, i.e., a deviation from normal but not associated with a disease state. In general, a cancer will be associated with the presence of one or more tumors, i.e., abnormal cell masses. The term “tumor” is meant to include all types of cancerous growths or oncogenic processes, metastatic tissues or malignantly transformed cells, tissues, or organs, irrespective of histopathologic type or stage of invasiveness. “Pathologic hyperproliferative” cells occur in disease states characterized by malignant tumor growth.

Tumors include malignancies of the various organ systems, such as affecting lung, breast, thyroid, lymphoid, gastrointestinal, and genito-urinary tract, as well as adenocarcinomas which include malignancies such as most colon cancers, renal-cell carcinoma, prostate cancer and/or testicular tumors, non-small cell carcinoma of the lung, hepatocellular cancer, cancer of the small intestine and cancer of the esophagus. The term “carcinoma” is art recognized and refers to malignancies of epithelial or endocrine tissues including respiratory system carcinomas, gastrointestinal system carcinomas, genitourinary system carcinomas, testicular carcinomas, breast carcinomas, prostatic

carcinomas, endocrine system carcinomas, and melanomas. In some embodiments, the disease is renal carcinoma or melanoma. Exemplary carcinomas include those forming from tissue of the cervix, prostate, breast, head and neck, colon and ovary. The term also includes carcinosarcomas, e.g., which include malignant tumors composed of carcinomatous and sarcomatous tissues. An "adenocarcinoma" refers to a carcinoma derived from glandular tissue or in which the tumor cells form recognizable glandular structures. The term "sarcoma" is art recognized and refers to malignant tumors of mesenchymal derivation.

Additional examples of cancers that can be treated using the methods and compositions described herein include brain and nervous system cancers, including, but not limited to, gliomas, glioblastomas, glioblastoma multiforme (GBM), oligodendrogliomas, primitive neuroectodermal tumors, low, mid and high grade astrocytomas, ependymomas (e.g., myxopapillary ependymoma papillary ependymoma, subependymoma, anaplastic ependymoma), oligodendrogliomas, medulloblastomas, meningiomas, pituitary adenomas, neuroblastomas, neurofibromas, malignant peripheral nerve sheath tumors, schwannomas, and craniopharyngiomas.

Additional examples of proliferative disorders include hematopoietic neoplastic disorders. As used herein, the term "hematopoietic neoplastic disorders" includes diseases involving hyperplastic/neoplastic cells of hematopoietic origin, e.g., arising from myeloid, lymphoid or erythroid lineages, or precursor cells thereof. For example, the diseases arise from poorly differentiated acute leukemias, e.g., erythroblastic leukemia and acute megakaryoblastic leukemia. Additional exemplary myeloid disorders include, but are not limited to, acute promyeloid leukemia (APML), acute myelogenous leukemia (AML) and chronic myelogenous leukemia (CML) (reviewed in Vaickus (1991) Crit Rev. in Oncol./Hemotol. 11:267-97); lymphoid malignancies include, but are not limited to acute lymphoblastic leukemia (ALL) which includes B-lineage ALL and T-lineage ALL, chronic lymphocytic leukemia (CLL), prolymphocytic leukemia (PLL), hairy cell leukemia (HLL) and Waldenstrom's macroglobulinemia (WM). Additional forms of malignant lymphomas include, but are not limited to non-Hodgkin lymphoma and variants thereof, peripheral T cell lymphomas, adult T cell leukemia/lymphoma (ATL),

cutaneous T-cell lymphoma (CTCL), large granular lymphocytic leukemia (LGF), Hodgkin's disease and Reed-Sternberg disease.

It is understood that the suitable dosage of an immunogen described herein will depend upon the age, sex, health, and weight of the recipient, the kind of concurrent treatment, if any, the frequency of treatment, and the nature of the effect desired. However, the most preferred dosage can be tailored to the individual subject, as determined by the researcher or clinician. The total dose required for any given treatment will commonly be determined with respect to a standard reference dose as set by a manufacturer, such as is commonly done with vaccines, such dose being administered either in a single treatment or in a series of doses, the success of which will depend on the production of a desired immunological result (i.e., successful production of a CTL-mediated response to the antigen, which response gives rise to the inhibition and/or treatment desired). Thus, the overall administration schedule must be considered in determining the success of a course of treatment and not whether a single dose, given in isolation, would or would not produce the desired immunologically therapeutic result or effect.

The therapeutically effective amount of a composition containing one or more of the immunogens described herein, is an amount sufficient to induce an effective CTL response to inhibit or arrest disease progression. Thus, this dose will depend, among other things, on the identity of the immunogens used, the nature of the disease condition, the severity of the disease condition, the extent of any need to prevent such a condition where it has not already been detected, the manner of administration dictated by the situation requiring such administration, the weight and state of health of the individual receiving such administration, and the sound judgment of the clinician or researcher. Thus, for purposes of prophylactic or therapeutic administration, effective amounts would generally lie within the range of from 1.0 μg to about 5,000 μg of peptide for a 70 kg patient, followed by boosting dosages of from about 1.0 μg to about 1,000 μg of peptide pursuant to a boosting regimen over days, weeks or months, depending on the recipient's response and as necessitated by subsequent monitoring of CTL-mediated activity within the bloodstream. Of course, such dosages are to be considered only a general guide and, in a given situation, the actual dosage can exceed such suggested dosage regimens where

the clinician believes that the recipient's condition warrants a more aggressive administration schedule. The efficacy of administering additional doses, and of increasing or decreasing the interval, can be re-evaluated on a continuing basis, in view of the recipient's immunocompetence (for example, the level of CTL activity with respect to tumor-associated or tumor-specific antigens).

For such purposes, the immunogenic compositions described herein can be used against a disease condition such as cancer by administration to an individual by a variety of routes. The compositions can be administered parenterally or orally, and, if parenterally, either systemically or topically. Parenteral routes include subcutaneous, intravenous, intradermal, intramuscular, intraperitoneal, intranasal, transdermal, or buccal routes. One or more such routes can be employed. Parenteral administration can be, for example, by bolus injection or by gradual perfusion over time.

Typically, vaccines are prepared as injectables, in the form of aqueous solutions or suspensions. Vaccines in an oil base are also well known such as for inhaling. Solid forms that are dissolved or suspended prior to use can also be formulated. Pharmaceutical carriers, diluents and excipients are generally added that are compatible with the active ingredients and acceptable for pharmaceutical use. Examples of such carriers include, but are not limited to, water, saline solutions, dextrose, or glycerol. Combinations of carriers can also be used. These compositions can be sterilized by conventional, well known sterilization techniques including sterile filtration. The resulting solutions can be packaged for use as is, or the aqueous solutions can be lyophilized, the lyophilized preparation being combined with sterile water before administration. Vaccine compositions can further incorporate additional substances to stabilize pH, or to function as adjuvants, wetting agents, or emulsifying agents, which can serve to improve the effectiveness of the vaccine.

The concentration of the CTL stimulatory peptides described herein in pharmaceutical formulations are subject to wide variation, including anywhere from less than 0.01% by weight to as much as 50% or more. Factors such as volume and viscosity of the resulting composition must also be considered. The solvents, or diluents, used for such compositions include water, dimethylsulfoxide, PBS (phosphate buffered saline), or saline itself, or other possible carriers or excipients.

The immunogens described herein can also be contained in artificially created structures such as liposomes, ISCOMS, slow-releasing particles, and other vehicles which increase the immunogenicity and/or half-life of the peptides or polypeptides in serum. Liposomes include emulsions, foams, micelles, insoluble monolayers, liquid crystals, phospholipid dispersions, lamellar layers and the like. Liposomes for use in the methods and compositions described herein are formed from standard vesicle-forming lipids which generally include neutral and negatively charged phospholipids and a sterol, such as cholesterol. The selection of lipids is generally determined by considerations such as liposome size and stability in the blood. A variety of methods are available for preparing liposomes as reviewed, for example, by Coligan, J. E. et al, Current Protocols in Protein Science, 1999, John Wiley & Sons, Inc., New York and U.S. Pat. Nos. 4,235,871, 4,501,728, 4,837,028, and 5,019,369. Liposomes containing the peptides or polypeptides described herein can be directed to the site of lymphoid cells where the liposomes then deliver the selected immunogens directly to antigen presenting cells. Targeting can be achieved by incorporating additional molecules such as proteins or polysaccharides into the outer membranes of said structures, thus resulting in the delivery of the structures to particular areas of the body, or to particular cells within a given organ or tissue. Such targeting molecules can include a molecule that binds to receptor on antigen presenting cells. For example an antibody that binds to CD80 could be used to direct liposomes to dendritic cells.

The immunogens described herein can also be administered as solid compositions. Conventional nontoxic solid carriers including pharmaceutical grades of mannitol, lactose, starch, magnesium, cellulose, glucose, sucrose, sodium saccharin, and the like. Such solid compositions will often be administered orally, whereby a pharmaceutically acceptable nontoxic composition is formed by incorporating the peptides and polypeptides described herein with any of the carriers listed above. Generally, such compositions will contain 10-95% active ingredient, and more preferably 25-75% active ingredient.

Aerosol administration is also an alternative, requiring only that the immunogens be properly dispersed within the aerosol propellant. Typical percentages of the peptides or polypeptides described herein are 0.01%-20% by weight, e.g., 1%-10%. The use of a

surfactant to properly disperse the immunogen may be required. Representative surfactants include the esters or partial esters of fatty acids containing from 6 to 22 carbon atoms, such as caproic, octanoic, lauric, palmitic, stearic, linoleic, linolenic, olesteric and oleic acids with an aliphatic polyhydric alcohol or its cyclic anhydride. Mixed esters, such as mixed or natural glycerides can be employed. The surfactant can constitute 0.1-20% by weight of the composition, e.g., 0.25-5%. Typical propellants for such administration can include esters and similar chemicals but are by no means limited to these. A carrier, such as lecithin, for intranasal delivery can also be included.

The peptides and polypeptides described herein can also be delivered with an adjuvant. Adjuvants include, but are not limited to, Toll-like receptor (TLR) agonists, Bacillus Calmette Guern (BCG), complete or incomplete Freund's adjuvant, a cytosine guanine oligodeoxynucleotide (CpG-ODN), Montanide ISA-51, Activation Gene-3 (LAG-3), aluminum phosphate, aluminum hydroxide, alum, and saponin. Adjuvant effects can also be obtained by administering one or more cytokines along with the immunogens described herein. These cytokines include, but are not limited to IL-1, IL-2, IL-7, IL-12, IL-13, IL-15, IL-18, and GM-CSF. Exemplary TLR agonists are described in Ghosh et al., 2006, Cell. Immunol., 243:48-57 and Lippincott's Illustrated Reviews: Immunology, Lippincott Williams & Wilkins; (July 1, 2007), ISBN-10: 0781795435, page 17.

The peptides and polypeptides described herein can also be added to professional antigen presenting cells such as dendritic cells that have been prepared ex vivo. For example, the dendritic cells could be prepared from CD34 positive stem cells from the bone marrow, or they could be prepared from CD14 positive monocytes obtained from the peripheral blood. The dendritic cells are generated ex vivo using cytokines such as GM-CSF, IL-3, IL-4, TNF, and SCF. The cultured DC are then pulsed with peptides at various concentrations using standard methods that are well known in the art. The peptide-pulsed dendritic cells can then be administered either intravenously, subcutaneously, or intradermally, and the immunization can also include cytokines such as IL-2 or IL-12.

An antigen presenting cell (APC)-based cancer vaccine can be delivered to a patient or test animal by any suitable delivery route, which can include injection,

infusion, inoculation, direct surgical delivery, or any combination thereof. In some embodiments, the cancer vaccine is administered to a human in the deltoid region or axillary region. For example, the vaccine is administered into the axillary region as an intradermal injection. In other embodiments, the vaccine is administered intravenously.

An appropriate carrier for administering APCs can be selected by one of skill in the art by routine techniques. For example, the pharmaceutical carrier can be a buffered saline solution, e.g., cell culture media, and can include DMSO for preserving cell viability.

The quantity of APCs appropriate for administration to a patient as a cancer vaccine can be based upon a variety of factors, as can the formulation of the vaccine itself. Some of these factors include the physical characteristics of the patient (e.g., age, weight, and sex), the physical characteristics of the tumor (e.g., location, size, rate of growth, and accessibility), and the extent to which other therapeutic methodologies (e.g., chemotherapy, and beam radiation therapy) are being implemented in connection with an overall treatment regimen. Notwithstanding the variety of factors one should consider in implementing the methods described herein to treat a disease condition, a mammal can be administered with from about 10^5 to about 10^8 APCs (e.g., 10^7 APCs) in from about 0.05 mL to about 2 mL solution (e.g., saline) in a single administration. Additional administrations can be carried out, depending upon the above-described and other factors, such as the severity of tumor pathology. In one embodiment, from about one to about five administrations of about 10^6 APCs is performed at two-week intervals.

APC vaccination can be accompanied by other treatments. For example, a patient receiving APC vaccination can also be receiving chemotherapy, radiation, and/or surgical therapy concurrently. Methods of treating cancer using APC vaccination in conjunction with chemotherapy are described in Wheeler et al., US Pat. Pub. No. 2007/0020297, the disclosure of which is incorporated herein by reference in its entirety. In some embodiments, a patient receiving DC vaccination has already received chemotherapy, radiation, and/or surgical treatment for the cancer. In one embodiment, a patient receiving DC vaccination is treated with a COX-2 inhibitor, as described in Yu and Akasaki, WO 2005/037995 and US 2008/0199484, the disclosure of each being incorporated herein by reference in its entirety.

The present invention is also directed to a vaccine in which an immunogen described herein is delivered or administered in the form of a polynucleotide encoding a polypeptide or fragment as disclosed herein, whereby the peptide or polypeptide or fragment is produced in vivo. The polynucleotide can be included in a suitable expression vector and combined with a pharmaceutically acceptable carrier. For example, the peptides or polypeptides could be expressed in plasmid DNA and nonreplicative viral vectors such as vaccinia, fowlpox, Venezuelan equine encephalitis virus, adenovirus, or other RNA or DNA viruses. These examples are meant to be illustrative only and should not be viewed as limiting. A wide variety of other vectors is available and are apparent to those skilled in the art from the description given herein. In this approach, a portion of the nucleotide sequence of the viral vector is engineered to express the peptides or polypeptides described herein. Vaccinia vectors and methods useful in immunization protocols are described in U.S. Pat. No. 4,722,848, the disclosure of which is incorporated herein by reference in its entirety.

Regardless of the nature of the composition given, additional therapeutic agents can also accompany the immunogens described herein. Thus, for purposes of treating tumors, compositions containing the immunogens disclosed herein can, in addition, contain other antitumor pharmaceuticals. The use of such compositions with multiple active ingredients is left to the discretion of the clinician.

A further embodiment of the present invention relates to a method for inducing a CTL response in a subject comprising administering to subjects that express HLA-A1, -A2 or -A3 supertype antigens an effective (i.e., CTL-stimulating) amount of an immunogen described herein, e.g., an amount sufficient to induce a CTL response to tumor cells expressing at least HLA-A1, HLA-A2, or HLA-A3, as the case may be, thereby eliciting a cellular response against said tumor cells.

A still further embodiment of the present invention relates to a method for inducing a CTL response in a subject, wherein the immunogen is in the form of a polynucleotide. In one non-limiting example, the method includes administering to subjects that express HLA-A1, HLA-A2, or HLA-A3 at least one CTL epitope, wherein said epitope or epitopes are selected from a group comprising the peptides described herein, and are coded within a polynucleotide sequence that does not include the entire

protein coding region, in an amount sufficient to induce a CTL response to tumor cells expressing HLA-A1, HLA-A2, or HLA-A3.

Antibodies

The immunogens described herein can be used to stimulate the production of antibodies for use in passive immunotherapy, for use as diagnostic reagents, and for use as reagents in other processes such as affinity chromatography.

The present invention also relates to antibodies that react with immunogens, such as a polypeptide comprising one or more of the epitopic peptides of SEQ ID NOs:1-21 (or a variant thereof) as described herein. Active fragments of such antibodies are also specifically contemplated. Such antibodies, and active fragments of such antibodies, for example, and Fab structure, can react with, including where it is highly selective or specific for, an immunogenic structure comprising 2, 3, 4 or more of the epitopic peptides described herein.

With the advent of methods of molecular biology and recombinant technology, it is now possible for the artisan of ordinary skill to produce antibody molecules by recombinant means and thereby generate gene sequences that code for specific amino acid sequences found in the polypeptide structure of the antibodies. Such antibodies can be produced by either cloning the gene sequences encoding the polypeptide chains of said antibodies or by direct synthesis of said polypeptide chains, with in vitro assembly of the synthesized chains to form active tetrameric (H_2L_2) structures with affinity for specific epitopes and antigenic determinants. This has permitted the ready production of antibodies having sequences characteristic of neutralizing antibodies from different species and sources.

Regardless of the source of the antibodies or nanobodies, or how the artisan of ordinary skill chooses to produce such antibodies or nanobodies, including recombinantly constructed or synthesized, in vitro or in vivo, by using transgenic animals, such as cows, goats and sheep, or by using cell cultures in bioreactors, or by direct chemical synthesis employing no living organisms at any stage of the process, all antibodies and nanobodies have regions capable of interacting with a structurally complementary antigenic target. The regions interacting with the target are referred to as "variable" or "V" regions and are

characterized by differences in amino acid sequence from antibodies of different antigenic specificity.

The antibodies can also be wholly synthetic, wherein the polypeptide chains of the antibodies are synthesized and, possibly, optimized for binding to the polypeptides disclosed herein as being receptors. Such antibodies can be chimeric or humanized antibodies and can be fully tetrameric in structure, or can be dimeric and include only a single heavy and a single light chain. Such antibodies can also include fragments, such as Fab and $F(ab')_2$ fragments, capable of reacting with and binding to any of the polypeptides disclosed herein as being receptors.

Superagonist Peptides

The peptides and immunogens disclosed herein can also include internal mutations that render them "superantigens" or "superagonists" for T cell stimulation. Superantigen peptides can be generated by screening T cells with a positional scanning synthetic peptide combinatorial library (PS-CSL) as described in Pinilla et al., 1992, Biotechniques, 13:901-5; Borras et al., 2002, J. Immunol. Methods, 267:79-97; US 2004/0072246; and Lustgarten et al.., 2006, J. Immun., 176:1796-1805. When a native T cell epitope is known, approximately 25% of the identified variants are found to be superagonists. These can be up to 3 orders of magnitude more effective than the native ligand (Hemmer et al., 2000, J. Immunol., 164: 861-871; La Rosa et al., 2001, Blood, 97:1776-86).

Positional scanning synthetic combinatorial libraries (PS-SCLs) representing trillions of peptides of different lengths can be used as unbiased sources of peptide antigens in T cell activation assays for the identification of T cell epitopes. PS-SCL (Pinilla et al., 1992, Biotechniques, 13:901-905) are composed of systematically arranged mixtures. In the case of a single position defined PS-SCL, each compound present in a given mixture has a common individual amino acid at a given position, while the remaining positions are composed of mixtures of all 19 natural L- amino acids (cysteine omitted). The screening data of a given PS-SCL permits the identification of key residues at each position of the peptide. It is important to note, however, that the activity found for a mixture is due to the presence of specific active peptide(s) within the mixture,

and not to the individual amino acids as separate entities. The combination of all amino acids defined in the most active mixtures leads to the active individual compounds.

Monitoring

The antigen-specific cellular immune responses of vaccinated subjects can be monitored by a number of different assays, such as tetramer assays, ELISPOT, and quantitative PCR. The following sections provide examples of protocols for detecting responses with these techniques. Additional methods and protocols are available. See e.g., Current Protocols in Immunology, Coligan, J. et al., Eds., (John Wiley & Sons, Inc.; New York, N.Y.).

Tetramers comprised of recombinant MHC molecules complexed with peptide can be used to identify populations of antigen-specific T cells. To detect T cells specific for antigens such as CD133, fluorochrome labeled specific peptide tetramer complexes (e.g., phycoerythrin (PE)-tHLA) containing peptides from these antigens are synthesized and provided by Beckman Coulter (San Diego, California). Specific CTL clone CD8 cells are resuspended at 10^5 cells/50 µl FACS buffer (phosphate buffer plus 1% inactivated FCS buffer). Cells are incubated with 1 µl tHLA for 30 minutes at room temperature and incubation is continued for 30 minutes at 4 °C with 10 µl anti-CD8 mAb (Becton Dickinson, San Jose, CA). Cells are washed twice in 2 ml cold FACS buffer before analysis by fluorescence-activated cell sorting (FACS) (Becton Dickinson).

ELISPOT assays can be used to detect cytokine secreting cells, e.g., to determine whether cells in a vaccinated patient secrete cytokine in response to antigen, thereby demonstrating whether antigen-specific responses have been elicited. ELISPOT assay kits are supplied from R & D Systems (Minneapolis, Minnesota) and performed as described by the manufacturer's instructions. Responder (R) 1×10^5 patients' PBMC cells from before and after vaccination are plated in 96-well plates with nitrocellulose membrane inserts coated with capture Ab. Stimulator (S) cells (TAP-deficient T2 cells pulsed with antigen) are added at the R:S ratio of 1:1. After a 24-hour incubation, cells are removed by washing the plates 4 times. The detection Ab is added to each well. The plates are incubated at 4 °C overnight and the washing steps will be repeated. After a 2-hour incubation with streptavidin-AP, the plates are washed. Aliquots (100 µl) of

BCIP/NBT chromogen are added to each well to develop the spots. The reaction is stopped after 60 min by washing with water. The spots are scanned and counted with computer-assisted image analysis (Cellular Technology Ltd, Cleveland, Ohio). When experimental values are significantly different from the mean number of spots against non-pulsed T2 cells (background values), as determined by a two-tailed Wilcoxon rank sum test, the background values are subtracted from the experimental values.

Quantitative PCR is another means for evaluating immune responses. To examine IFN-γ production in patients by quantitative PCR, cryopreserved PBMCs from patients' pre-vaccination and post-vaccinations samples and autologous dendritic cells are thawed in RPMI DC culture medium with 10% patient serum, washed and counted. PBMC are plated at 3 x 10^6 PBMCs in 2 ml of medium in 24-well plate; dendritic cells are plated at 1x10^6/ml and are pulsed 24 hour with 10 μg/ml tumor peptide in 2 ml in each well in 24 well plate. Dendritic cells are collected, washed, and counted, and diluted to 1x 10^6 /ml, and 3x10^5 (i.e., 300 μl solution) added to wells with PBMC (DC: PBMC=1:10). 2.3 μl IL-2 (300 IU/mL) is added every 3-4 days, and the cells are harvested between day 10 and day 13 after initiation of the culture. The harvested cells are then stimulated with tumor cells or autologous PBMC pulsed with 10 μg/ml tumor peptide for 4 hours at 37 °C. On days 11-13, cultures are harvested, washed twice, then divided into four different wells, two wells using for control (without target); and another two wells CTL co-cultured with tumor cells (1:1) if tumor cells are available. If tumor cells are not available, 10 μg/ml tumor lysate is added to CTL. After 4 hours of stimulation, the cells are collected, RNA extracted, and IFN-γ and CD8 mRNA expression evaluated with a thermocycler/fluorescence camera system. PCR amplification efficiency follows natural log progression, with linear regression analyses demonstrating correlation coefficients in excess of 0.99. Based on empirical analysis, a one-cycle difference is interpreted to be a two-fold difference in mRNA quantity, and CD8-normalized IFN-γ quantities are determined. An increase of > 1.5-fold in post-vaccine relative to pre-vaccine IFN-γ is the established standard for positive type I vaccine responsiveness.

Ex Vivo Methods

The following protocol can be used to produce antigen-presenting cells and/or antigen-specific CTL in vitro from patient-derived PBMC. To generate dendritic cells, the plastic adherent cells from PBMCs are cultured in AIM-V medium supplemented with recombinant human GM-CSF and recombinant human IL-4 at 37 °C in a humidified CO_2 (5%) incubator. Six days later, the immature dendritic cells in the cultures are stimulated with recombinant human TNF-α for maturation. Mature dendritic cells are then harvested on day 8, resuspended in PBS at 1 x 10^6 per mL with peptide (2 μg/mL), and incubated for 2 hours at 37 °C.

Autologous CD8+ T cells are enriched from PBMCs using magnetic microbeads (Miltenyi Biotech, Auburn, CA). CD8+ T cells (2 x 10^6 per well) are co-cultured with 2 x 10^5 per well peptide-pulsed dendritic cells in 2 mL/well of AIM-V medium supplemented with 5% human AB serum and 10 units/mL rhIL-7 (Cell Sciences) in each well of 24-well tissue culture plates. About 20 U/ml of IL-2 is added 24 h later at regular intervals, 2 days after each restimulation. On day 7, lymphocytes are restimulated with autologous dendritic cells pulsed with peptide in AIM-V medium supplemented with 5% human AB serum, rhIL-2, and rhIL-7 (10 units/mL each). About 20 U/ml of IL-2 is added 24 h later at regular intervals, 2 days after each restimulation. On the seventh day, after the three rounds of restimulation, cells are harvested and tested the activity of CTL. The stimulated CD8+ cultured cells (CTL) are co-cultured with T2 cells (a human TAP-deficient cell line) pulsed with 2 μg/ml CD133 peptides. After 24 hours incubation, IFN-γ in the medium is measured by ELISA assay.

Animal Models

Vaccination (e.g., DC vaccination) can be evaluated in animal models. Suitable models for cancers include injection models, in which cells of a tumor cell line are injected into the animal, and genetic models, in which tumors arise during development. In some cases, a transgenic animal (e.g., a mouse) that expresses an HLA (e.g., HLA-A2) can be used. See, e.g., Choi et al., 2002, J. Immunol. Methods, 268:35-41.

To evaluate dendritic cell vaccination in an animal model, functional dendritic cells are isolated from bone marrow derived cells of the animal and differentiated in vitro

in the presence of cytokines, as detailed above. Mature dendritic cells are pulsed with tumor antigens (e.g., tumor antigens derived from the tumor cell line that will be implanted into the animal or synthetic peptides corresponding to epitopes of those antigens). Animals are implanted with cells of the tumor cell line. After implantation, animals are vaccinated with antigen-pulsed dendritic cells one or more times. Survival and immune responsiveness is measured.

Kits

The present invention is also directed to kits to treat cancers. The kits are useful for practicing the methods described herein for treating cancer with a vaccine comprising an antigen or APCs loaded with an antigen as described herein. The kit is an assemblage of materials or components, including at least one of the compositions described herein. Thus, in some embodiments, the kit includes a set of peptides for use in vaccination or preparing cells for vaccination. The kit can also include agents for preparing cells (e.g., cytokines for inducing differentiation of DC in vitro). The invention also provides kits containing a composition including a vaccine comprising dendritic cells (e.g., cryopreserved dendritic cells) loaded with the antigens as described herein.

The exact nature of the components configured in the kit depends on its intended purpose. For example, some embodiments are configured for the purpose of treating brain cancer, colon cancer, hepatocellular carcinoma, prostate cancer, multiple myeloma, and melanoma. In one embodiment the brain cancer is a glioma. In another embodiment, the brain cancer is glioblastoma multiforme (GBM). In another embodiment, the brain cancer is an astrocytoma. In one embodiment, the kit is configured particularly for the purpose of treating mammalian subjects. In another embodiment, the kit is configured particularly for the purpose of treating human subjects. In further embodiments, the kit is configured for veterinary applications, treating subjects such as, but not limited to, farm animals, domestic animals, and laboratory animals.

Instructions for use can be included in the kit. "Instructions for use" typically include a tangible expression describing the technique to be employed in using the components of the kit to effect a desired outcome, such as to treat cancer. For example, the instructions can include instructions to administer a vaccine (e.g., comprising

dendritic cells loaded with the antigens described herein) to the patient. Instructions for use can also include instructions for repeated administrations of the vaccine; for example, administering the three doses of the vaccine in two week intervals.

Optionally, the kit also contains other useful components, such as, diluents, buffers, pharmaceutically acceptable carriers, syringes, catheters, applicators, pipetting or measuring tools, or other useful paraphernalia as will be readily recognized by those of skill in the art.

The materials or components assembled in the kit can be provided to the practitioner stored in any convenient and suitable ways that preserve their operability and utility. For example the components can be in dissolved, dehydrated, or lyophilized form; they can be provided at room, refrigerated or frozen temperatures. The components are typically contained in suitable packaging material(s). As employed herein, the phrase "packaging material" refers to one or more physical structures used to house the contents of the kit, such as compositions described herein and the like. The packaging material is constructed by well known methods, preferably to provide a sterile, contaminant-free environment. The packaging materials employed in the kit are those customarily utilized in cancer treatments or in vaccinations. As used herein, the term "package" refers to a suitable solid matrix or material such as glass, plastic, paper, foil, and the like, capable of holding the individual kit components. Thus, for example, a package can be a glass vial used to contain suitable quantities of a composition containing a vaccine, e.g., a vaccine comprising an immunogen or dendritic cells loaded with the antigens as described herein. The packaging material generally has an external label which indicates the contents and/or purpose of the kit and/or its components.

EXAMPLES

The following Examples are illustrative and not limiting.

Example 1. Prediction and Synthesis of CD133 Epitopes

Epitopes of CD133 were predicted using the Immune Epitope free public database for predicting Class 1 MHC-peptide binding to HLA-A*0101, -A*0201 and -A*0301 using the artificial neural network (ANN) method (Nielsen et al., 2003, Protein Sci.,

12:1007-17). The algorithm used be found on the World Wide Web at tools.immuneepitope.org/analyze/html/mhc_binding.html. Peptides were selected based on predicted IC_{50} for HLA-A*0101 (IC50 $\leq$ 5000 nM), HLA-A*0201 (IC50 $\leq$ 500 nM), and HLA-A*0301 (IC50 $\leq$ 500 nM). Eighty-four candidate nine-amino acid sequences of CD133 were synthesized (ProImmune Ltd., Oxford, UK) to determine whether the peptides bound to the MHCs HLA-A*0101, HLA-A*0201, and HLA-A*0301. The information generated from the Immune Epitope Database is only a general guideline. These scores are based solely on algorithms and cannot confirm whether the sequences are true or optimal T cell epitopes. Additionally, there may be some sequences that do not score well on algorithms that will be good T cell epitopes.

Example 2. Identification of HLA-A*0101 Epitopes

Eighteen of the candidate peptides were assembled with HLA-A*0101 and analyzed for MHC binding to determine their level of incorporation into MHC molecules using the REVEAL™ MHC binding assay (ProImmune Ltd., Oxford, UK). Binding to MHC molecules was compared to that of two known T-cell epitopes: an intermediate control peptide and a positive control peptide with weak and very strong binding properties, respectively. Three peptides bound to HLA-A*0101 at at least 50% of the level of the positive control, as indicated on Table 1.

Table 1. HLA-A*0101 Binding

SEQ ID NO	Sequence	% Positive Control
1	AVDVFLCSY	58.58
2	SSELESLKV	74.14
3	IIDPLNLFW	91.46
Intermediate Control	-	2.25 ± 1.2
Positive Control	-	100.00 ± 15.2

The peptides that bound to HLA-A*0101 were synthesized as ProVE® pentamers (ProImmune Ltd., Oxford, UK) for further analysis. The pentamers were subjected to

incubation and analysis at 37 °C to determine the stability of peptide-MHC complexes. Off-rates of the complexes were measured by REVEAL™ off rate analysis (ProImmune Ltd., Oxford, UK) after 0, 2,and 24 hours of incubation at 37 °C. The off-rates of the peptides in terms of $t_{1/2}$ half-life values are presented in Table 2.

Table 2. HLA-A*0101 Off Rates

SEQ ID NO	Sequence	$t_{1/2}$ (h)
1	AVDVFLCSY	20.71
2	SSELESLKV	0.46
3	IIDPLNLFW	0.35
Intermediate Control	-	0.29
Positive Control	-	102.74*

*: The measurement interval of 24 hours was too short to calculate this value accurately.

Example 3. Identification of HLA-A*0301 Epitopes

Fifteen of the candidate peptides were assembled with HLA-A*0301 and analyzed for MHC binding to determine their level of incorporation into MHC molecules using the REVEAL™ MHC binding assay (ProImmune Ltd., Oxford, UK). Binding to MHC molecules was compared to that of two known T-cell epitopes: an intermediate control peptide and a positive control peptide with weak and very strong binding properties, respectively. Six peptides bound to HLA-A*0301 at at least 45% of the level of the positive control, as indicated on Table 3.

Table 3. HLA-A*0301 Binding

SEQ ID NO	Sequence	% Positive Control
4	KLFNKSKMK	80.81
5	ILAQYNTTK	46.61
6	YLSGKLFNK	46.87
7	RTRIKRSRK	87.33

8	LSSSLTSVK	52.49
9	NLFWFGIGK	53.08
Intermediate Control	-	9.19 ± 1.5
Positive Control	-	100.00 ± 4.6

Five of the peptides that bound to HLA-A*0301 were synthesized as ProVE® pentamers (ProImmune Ltd., Oxford, UK) for further analysis. The pentamers were subjected to incubation and analysis at 37 °C to determine the stability of peptide-MHC complexes. Off-rates of the complexes were measured by REVEAL™ off rate analysis (ProImmune Ltd., Oxford, UK) after 0, 2,and 24 hours of incubation at 37 °C. The off-rates of the peptides in terms of $t_{1/2}$ half-life values are presented in Table 4.

Table 4. HLA-A*0301 Off Rates

SEQ ID NO	Sequence	$t_{1/2}$ (h)
4	KLFNKSKMK	27.81*
5	ILAQYNTTK	2.07
7	RTRIKRSRK	23.92
8	LSSSLTSVK	1.80
9	NLFWFGIGK	3.35
Intermediate Control	-	0.56
Positive Control	-	49.82*

*: The measurement interval of 24 hours was too short to calculate these values accurately.

Example 4. Identification of HLA-A*0201 Epitopes

Fifty-five of the candidate peptides were assembled with HLA-A*0201 and analyzed for MHC binding to determine their level of incorporation into MHC molecules using the REVEAL™ MHC binding assay (ProImmune Ltd., Oxford, UK). Binding to MHC molecules was compared to that of two known T-cell epitopes: an intermediate

control peptide and a positive control peptide with weak and very strong binding properties, respectively. Eleven peptides bound to HLA-A*0201 as well or better than the positive control, as indicated on Table 5.

Table 5. HLA-A*0201 Binding

SEQ ID NO	Sequence	% Positive Control
10	VLDEIKSMA	309.26
11	YLQWIEFSI	112.67
12	NLLSFAYDL	179.71
13	FITNNTSSV	107.15
14	RVLDTPYLL	165.18
15	SLDFAQNFI	114.58
16	ELVHIFLYV	155.93
17	LVLGSLLLL	101.90
18	SQLNSNPEL	123.10
19	ILCCVLGLL	163.06
20	GLLERVTRI	131.30
21	FLLPALIFA	93.59
Intermediate Control	-	2.65 ± 2.1
Positive Control	-	100.00 ± 6.7

Twelve of the peptides that bound to HLA-A*0201 were synthesized as ProVE® pentamers (ProImmune Ltd., Oxford, UK) for further analysis. The pentamers were subjected to incubation and analysis at 37 °C to determine the stability of peptide-MHC complexes. Off-rates of the complexes were measured by REVEAL™ off rate analysis (ProImmune Ltd., Oxford, UK) after 0, 2,and 24 hours of incubation at 37 °C. The off-rates of the peptides in terms of $t_{1/2}$ half-life values are presented in Table 6.

Table 6. HLA-A*0201 Off Rates

SEQ ID NO	Sequence	$t_{1/2}$ (h)
10	VLDEIKSMA	1.43
11	YLQWIEFSI	83.00*
12	NLLSFAYDL	1.83
13	FITNNTSSV	19.98
14	RVLDTPYLL	3.51
15	SLDFAQNFI	15.49
16	ELVHIFLYV	6.86
17	LVLGSLLLL	8.25
18	SQLNSNPEL	2.37
19	ILCCVLGLL	5.63
20	GLLERVTRI	74.30*
21	FLLPALIFA	> 120*
Intermediate Control	-	16.18
Positive Control	-	55.67*

*: The measurement interval of 24 hours was too short to calculate these values accurately.

Example 5. Generation of Superagonist CD133 Peptides

Superagonist peptides of the CD133 epitopes described herein are produced by the methods described below. These peptide superagonists exhibit a superior capacity to induce CTL responses.

For this application T cell lines and clones are generated from peripheral blood mononuclear cells (PBMC) derived from glioma patients. Epstein Barr transformed autologous B cells are used as antigen presenting cells through all the T cell functional assays and stimulations. Blood is obtained from glioma patients and carefully layered on top of 50 ml conical tubes (polypropylene, Sarsted) in a ratio of 2 volumes per 1 volume of Histopaque (Sigma, St Louis, Missouri). Each tube is then placed in a clinical swing out centrifuge (Beckman) and spun down for 30 minutes at 400 g at room temperature.

The PBMC are then collected from the interface with a transfer plastic pipette (Samco) and washed 2x with D-PBS at 250 g and 1x with culture medium (IMDM, Bio-whittaker, Walkersville, Maryland) containing 8% AB human serum (Gemini Bio-products, Woodland, California) at 200 g for 10 minutes each step. The supernatant is aspirated and discarded, and the cells are resuspended in culture medium.

CD8+ and CD4+ T cells are isolated from PBMC by positive selection following manufacturer's instructions (CD8 and CD4 positive selection kits, Dynal Biotech Inc., Lake Success, NY). The isolated cells are used immediately for stimulation protocols.

Transformation of B cells from PBMC by Epstein Barr virus (EBV) is performed immediately after PBMC isolation. Briefly, frozen PBMCs are thawed, washed, and resuspended in CRPMI 10% FBS. 5 to 10 million PBMCs are resuspended in 2.5 ml of CRPMI 10% FBS. Then, 2.5 ml of thawed supernatant from B95.8 Marmoset cells (containing the EBV) are added to each conical tube containing the cells. The cells are incubated for 2 hours in a water bath at 37 °C. CRPMI 10% FBS containing 1 μg/ml of Cyclosporin A is then added to each tube. 10 ml suspensions are transferred to T-25 flasks and incubated for 3 weeks. At this point, the cells form clumps visible to the naked eye. By microscopic examination, the cells appear large, clear and possibly hairy. These are indicators of B cell immortalization by EBV. Cells are mixed in their flasks and the 10ml culture is split into 2 new T-25 flasks (5 ml each). 5 ml of fresh CRPMI-10 media containing 1 μg/ml cyclosporin A is added to each flask and the cultures are incubated for 1 week at 37 °C. At this time point, an aliquot of each donor's cells is stained for CD19 expression (Pharmingen anti-CD19-APC stain) and analyzed by flow cytometry. The cell lines are then expanded and frozen down at 5 x 10^6/vial. Immortalized B cells are expanded in culture by splitting 1:3 in CRPMI-10 media (without cyclosporin A) in T-25 flasks once a week and incubating at 37 °C, 5% CO_2. These lymphoblastoid B cell lines (EBV-LCL) are used as antigen presenting cells in the following T cell functional assays.

PBMC are stimulated with the reported CD133 antigens and with cancer stem cell lines in the presence of autologous dendritic cells. Briefly, T cells derived from either single well or multiple wells (bulk cultures) are used after 6-7 days of stimulation. T cell limiting dilutions are done at a concentration of 0.3, 1, 3 and 10 cells/well in 96-well

round bottom plates (Corning). 1 x 10^5 irradiated autologous dendritic cells per well are added together with IL-2 and IL-7 (20 U/ml and 10 ng/ml, respectively). About five to ten times the original number of the plated cells is obtained. Wells that demonstrate growth are expanded by restimulation with a larger number of irradiated allogeneic feeders, phytohemagglutinin (PHA), and IL-2 until sufficient numbers are obtained for specificity tests. At this point, some cells are frozen while others are tested for antigen reactivity by using different readouts of T cell activation, namely cytokine production, cell killing and proliferation. Multiplex cytokine assay (Millipore, Billerica, MA) is performed according to the manufacturer's instructions to quantify, in an unbiased manner, a large cytokine spectrum to determine the best cytokine(s) for the evaluation of antigen specificity.

TCR profiles of the generated T cell clones are obtained to demonstrate and monitor clonality. The Vβ repertoire is determined using flow cytometry (as described above) with specific mAbs (available through Immunotech, Miami, Florida) for cells that expand to large numbers (> 10 million).

Immortalization of the antigen-responsive human T cells from PBMCs provides an advantage for the study of their fine specificity with the combinatorial libraries, because a high number of T cells are needed for the screening of the these libraries. Indeed, in order to obtain adequate data from combinatorial libraries, cells should be grown to a minimal of 30 to 100 million cells. For this reason, we the defined T cell lines and clones will be immortalized. Briefly, transduction is obtained by magnetofection in dividing T cells (recently stimulated), which are washed, counted, and plated with 100U/ml of IL-2 in complete medium in 96 well plates (flat bottom). A mixture of the retroviral vector with Viromag R/L (OZ Biosciences) is incubated for 20 minutes before being layered onto the T cells, and the plate is then carefully set on the top of magnetic plate and incubated overnight. The next day the cells are resuspended in fresh complete medium with IL-2 and transferred to a larger well. After 48 hours the transfection efficiency is assessed by flow cytometry by staining with anti-NGFR-PE. Magnetic bead enrichment of transduced cells is performed according to Miltenyi protocols using anti - PE beads (Miltenyi).

CD133-specific T cell lines and clones are obtained within 2-4 months from the primary stimulation.

Combinatorial peptide libraries for screening for superagonist peptides are prepared as described previously in Pinilla et al., 1994, Biochem. J., 301:847-853.

T cell functional assays are performed in 96-well plates (Corning Inc., Corning, NY). Each plate can accommodate 80 samples in columns 3-12, with the first 2 columns reserved for negative and positive control wells. The dispensing of samples and common reagents is accomplished using a Precision Biotek automated liquid handling instrument (Biotek, Winooski, Vermont). All samples, both libraries and individual compounds, are stored in 96-tube racks that are compatible with both the 96-well plates and the liquid handler instrumentation. Thirty plates per week are tested with the T cell functional assays. For assays that are run in duplicate, this generates approximately 1,000 data points per week. Data are acquired in the instruments specified for each type of assay and transferred to specifically designed Excel workbooks for rapid and accurate analysis.

Library mixtures are tested at a final concentration of 100 or 200 μg/ml using the general plate layout described above. Briefly, 25,000 T cells are cultured in the presence of 50,000 irradiated autologous lymphoblastoid cell lines (LCLs) and 25 μl of each mixture library at 2 mg/ml in complete RPMI. Each mixture is tested in duplicate. Control wells include T cells and LCLs without mixtures and with or without PHA (at a final concentration of 5ug/ml). As mentioned before for antigen specificity, different readouts of T cell activation are tested to confirm the assay readout that provides the best signal for the screening with the libraries. After the screening with the library, the results are used to design individual peptides by combining the selection of the defined amino acids of the most active mixtures at each defined position. This approach provides optimized agonists and superagonist peptides of the CD133 epitopes described herein. The most active peptides are selected to determine their in vitro immunogenicity and cross reactivity with the native antigen.

Individual agonist and superagonist peptides are synthesized by the simultaneous multiple peptide synthesis method (Houghten, 1985, Proc. Natl. Acad. Sci. USA, 82:5131-35). The purity and identity of each peptide are characterized using an electrospray mass spectrometer interfaced with a liquid chromatography system.

To test the stimulatory capacity of the peptides, 25,000 T cells are cultured in the presence of 50,000 irradiated autologous LCLs and each of the individual peptides at a final concentration of 10 and 1 μg/ml. The stimulatory activity of the positive peptides is determined with dose-titration experiments to determine the concentration that yields 50% stimulatory activity (EC-50).

These studies identify superagonist peptides derived from the CD133 described herein. Strong agonist peptides recognized with EC-50 values in the nanomolar range are identified.

Example 6. Immunization with CD133 Peptides

Vaccination with CD133 epitope peptides described herein and superagonists thereof is tested for killing of tumors in humanized HLA-A2 transgenic mice. Similar methods can also be performed in mice transgenic for other HLA (see, e.g., Alexander et al., 1997, J. Immunol., 159:4753-61). The efficacy of vaccination with CD133 epitope and its superagonists with regard to peripheral cytotoxicity, intracranial tumor infiltration, and survival is tested.

Briefly, HHD mice are immunized with an epitope peptide described herein emulsified in Incomplete Freund's adjuvant and helper antigen. Bulk populations of splenocytes are tested for specific cytotoxicity against the EL4-HHD cells pulsed with the peptide, control unpulsed EL4-HHD, or EL4-HHD- peptide cells. Measurement of the peptide/HLA complex binding and stability is performed. Survival of animals vaccinated with CD133 epitope superagonists is compared.

CD133 peptides and superagonists are synthesized by N-(9-fluorenyl) methoxycarbonyl chemistry at >95% purity as indicated by analytic high-performance liquid chromatography and mass spectrometric analysis. Peptides are dissolved in PBS/10% DMSO at a concentration of 2 mg/ml and stored at –20 °C until use.

The peptides are tested in HHD mice, which are humanized with regard to HLA-A2 expression (Pascolo et al., 1997, J. Exp. Med., 185:2043-51). The HHD mice used are Dbcβ2 microglobulin null and transgenic for modified HLA-A*0201-β2 microglobulin single chain (HHD) (Eguchi et al., 2006, Cancer Res., 66:5883-91; Gross et al., 2004, J. Clin. Invest., 113:425-433).

An HHD-syngeneic tumor cell line that expresses CD133 is created. The full-length human CD133 cDNA fragment is generated by reverse transcription-PCR using forward (AGTATGGCTTTCGTTTGCTTGGC; SEQ ID NO:22) and reverse (TACCGAGCTCGGATCCACTAGT; SEQ ID NO:23) primers and CSC1 glioblastoma multiforme cancer stem cell-derived total RNA. The CD133 cDNA is then cloned into the expression plasmid pEF6/V5-His-TOPO vector (Invitrogen) to generate pEF6/V5-CD133. EL4-HHD cells are then transfected with the pEF6/V5-CD133 using Cell Line Nucleofector kit T (Amaxa, Gaithersburg, Maryland), and a blasticidine-resistant clone that stably expresses the highest level of CD133 based on flow-cytometry using CD133 mAb (Tessa) is selected (EL4-HHD-CD133) for further use.

Cells are stained with phycoerythrin-conjugated HLA-A*0201/ peptide tetramers (10 μg/mL) in PBS containing 1% bovine serum albumin for 15 minutes at room temperature, washed once, and stained with FITC-conjugated anti-human CD8 or anti-mouse CD8 (BD Biosciences, San Diego, CA). Flow cytometric analyses are performed using Coulter EPICS cytometer (Beckman Coulter, Fullerton, Califorina).

To measure the peptide/HLA-A2 complex binding and stability, T2 cells (1×10^6 cells/mL) are incubated with various concentrations (0.1-100 nmol/L) of peptides in serum-free RPMI 1640 at 37 °C overnight in an atmosphere containing 5% CO_2. The cells are then washed twice with PBS and stained with the BB7.2 mAb for 30 minutes at 4 °C. After washing, FITC-conjugated goat anti-mouse IgG (Caltag, Burlingame, California) is used as the secondary antibody. Surface expression levels of HLA-A2 are examined by flow cytometry. Peptide binding is evaluated by determining mean fluorescence intensity (MFI).

HHD mice are vaccinated (on days 0 and 7) with s.c. injections of 100 μg of peptide or superagonist emulsified in Incomplete Freund's adjuvant (IFA; Difco, Detroit, Michigan) in the presence of 140 μg of the I-Ab-restricted HBVcore128 T-helper epitope, which stimulates a CD4+ helper T-cell response. Control animals receive IFA containing HBV helper-peptide only. On day 11 after the second immunization, the animals are sacrificed, and 5×10^7 splenocytes are stimulated in vitro with the same peptide that is used for in vivo stimulation (10 μmol/L). On day 6 of culture, the bulk

populations are tested for specific cytotoxicity against EL4-HHD or EL4-HHD-peptide cells.

To assess systemic protective immunity against i.c. tumor challenge, on day 7 after the second immunization, HHD mice receive an i.c. inoculation of EL4-HHD-peptide cells. Briefly, 5 x 104 EL4-HHD-peptide cells are stereotactically injected through an entry site at the bregma 2 mm to the right of the sagittal suture and 3 mm below the surface of the skull of anesthetized mice using a stereotactic frame. The animals are monitored daily after treatment for the manifestation of any pathologic signs.

Mice bearing i.c. EL4-HHD-peptide tumors receive immunizations on days 14 and 21 after the tumor inoculation, sacrificed by CO_2 asphyxiation on day 28, and perfused through the left cardiac ventricle with PBS. Brains are enzymatically digested (Walker et al., 2000, J. Immunol., 165 3128-35; Calzascia et al., 2005, Immunity, 22:175-184), and cells from each brain are resuspended in 70% Percoll (Sigma, Saint Louis, Missouri), overlaid with 37% and 30% Percoll and centrifuged for 20 minutes at 500 x g. Enriched brain-infiltrating lymphocyte (BIL) populations are recovered at the 70% to 37% Percoll interface.

Survival data are compared using a log-rank test. Comparative numbers of T-cell responses are analyzed by Student's t test for two samples with unequal variances. Statistical significance is determined at the < 0.05 level. Positive response is also defined as follows: the specific lysis by the responder cells against antigen-positive target cells is at least 15% and 2-fold higher than lytic levels by corresponding control conditions in at least two effector/target (E/T) ratios. Post-hoc contrasts (e.g., Student's 't' test) are performed to determine significant differences, i.e., $p < 0.05$ between the 3 groups of animals receiving epitope vaccination, control vaccinations, and PBS vehicle control. 10 animals /group are used, sufficient to detect a 1.2 SD difference between groups at a power of 0.8 and a $p = 0.05$.

Brain inflammation in response to vaccination is measured by performing a quantitative stereological analysis of the infiltration of T, B, and NK lymphocytes and macrophages. An immune cellular infiltrate is detected only in the intracranial tumor. Influx of CD4+, CD8+, and NK cells is observed within the tumor and peritumoral area.

Increased activation of astrocytes is also observed, as evidenced by up-regulation of GFAP immunoreactivity in astrocytes.

Example 7. Induction of Immune Responses Using CD133 Superagonist Peptides

The capacity of CD133 superagonist peptides to induce CTLs capable of cross-reacting against the wild-type epitope is determined. HLA binding and stability assays are performed to determine whether the improved immunogenicity of the analog peptides is at least partially attributable to higher binding/stability of these superagonist peptides in HLA complexes that are required for specific CTL recognition. CTL assays analyzing reactivity versus peptide dose titration on T2 target cells are performed to detect whether the CTLs developed using the superagonist peptides possesses a higher functional avidity than those primed with wild-type peptide. CTL clones raised against the agonistic peptide-epitope have a more restricted T cell receptor (TCR) usage and higher TCR functional avidity than the CTL clones raised against the natural peptide-epitope.

PBMCs are obtained from glioma patients and healthy donors under an Institutional Review Board–approved protocol. HLA (e.g., HLA-A2) expression on the PBMC is validated using the monoclonal antibodies (mAb) MA2.1 (against HLA A2, B17) and BB7.2 (against HLA A2, Aw69: both from the American Type Culture Collection, Manassas, Virginia) in indirect immunofluorescence assays monitored by flow cytometry.

HLA-A*0201 restricted CTL clones specific for CD133 natural and superagonist peptide-epitopes are generated in vitro. Harvested mature monocyte derived dendritic cells (mMoDC) are pulsed with natural and superagonist peptides (20 μM) and, after washing, are mixed with magnetically enriched CD8+ T cells from either thawed cryopreserved CD14 negative PBMCs or fresh PBMCs. Peptide pulsed mMoDC and enriched CD8+ T cells are mixed at a ratio of 1:20 in the presence of sCD40L (2 μg/ml) to initiate Th1-polarization of mMoDC, which boosts IL-12 production (Mailliard et al., 2002, J. Exp. Med., 195:473-483; Mailliard et al., 2004, Cancer Res., 64: 5934-37). On day three, the priming culture is supplemented with IL-2 (50 U/ml) and IL-7 (10 U/ml), and on day 12 the culture is restimulated with peptide pulsed autologous PBMC. At day

24-28, the priming culture is tested by tetramer staining for the presence of expanded primed CTL specific for the peptide used. As a positive control, priming with HLA-A*0201 restricted p24HIV-1 (SLYNVATL; SEQ ID NO:24) is run concurrently (Kan-Mitchell et al., 2006, J. Immunol., 176:6690-6701; Mitchell et al., 2007, Cancer Immunol. Immunother., 56:287-301).

For assessment of stability, patient-derived T2 cells (1 x 10^6 per mL) are incubated overnight with 100 μmol/L of each peptide in serum-free RPMI 1640 at 37 °C. Thereafter, the cells are washed four times to remove free peptides and incubated at 37 °C for 0, 3, or 6 hours. The cells are stained with the BB7.2 mAb to evaluate the HLA-A2 molecule expression at each time point. Peptide-induced HLA-A2 expression is evaluated by calculating the mean fluorescence of peptide-incubated T2 cells minus the mean fluorescence of T2 cells in the absence of peptide. DC50 is measured as the time required for the loss of 50% of the HLA-A2/peptide complexes stabilized at t = 0.

TCR usage of CTL clones is determined by expression of variable region of β chain (V- β) of TCR. Expression of TCR-V-β and V-α among clonally expanded CD8 T cells is assessed by a real-time PCR using a fluorogenic probe (Lang et al., 1997, J. Immunol. Methods, 203:181-192). This method offers a similar degree of sensitivity to the conventional detection of TCR- V- β expression with reduced processing time. Briefly, total RNA extraction and reverse transcription are performed. In the PCR step, a V-β-specific 5' probe, common CB 3' primers, and an internal fluorogenic probe are used to amplify 26 possible V-β genes. The detection and quantitation of PCR products are done by using a 7900HT Fast Real-Time PCR System (Applied Biosystems), with which it is possible to calculate the semi-quantitative ratio of TCR V-β expression among clonally expanded CD8 T cells. Once the expression of a particular TCR V- β is determined, using the same V- β specific primer the sequence corresponding to CDR 3 is determined. This allows for delineation of the clonality of CTLs.

Tetramer decay analysis is performed to determine TCR avidity of the CTL clones (Savage et al., 1999, Immunity, 10:485-492). CTL clones are stained with tetramer (1–25 nM), as in the equilibrium binding experiments above. Cells are washed twice with FACS buffer (4% FCS and 0.1% sodium azide in PBS) and kept on ice until they are mixed with excess anti-HLA-A02 mAb (BB7.2, BD Bioscienes) and then

incubated at room temperature to allow for tetramer dissociation. The anti-HLA-A02 mAb is used to block rebinding of tetramer to the TCR. Dissociation is followed for 0-180 minutes, after which cells are washed quickly with ice-cold buffer to remove all unbound tetramer and blocking mAb. The cells are then fixed for flow cytometry analysis (CyanADP, Beckman-Coulter). The natural logarithm of percentage of Geometric Mean Fluorescence (GMF) at each time point (compared with 0 minutes) is plotted against time. The half-life of each pMHC multimer is derived from the slope by the equation t1/2 - ln2/slope.

CTL activity of the in vitro primed CTL clones is measured by flow cytometric assay (Betts et al., 2003, J. Immunol. Methods, 281:65-78; Betts et al., 2004, Methods Cell Biol., 75:497-512). Briefly, the priming culture containing the CTL clone is mixed 1:1 with peptide pulsed T2 cells for 6 hours in the presence of CD107a, Monensin, and Brefeldin A. After the 6 hour incubation, cells are stained with corresponding tetramers and anti-CD8 mAb, followed by intracellular IFN-γ and TNFα staining. Stained cells are run on Beckman-Coulter CyanADP (9 color, 11 parameters) for flow cytometric analysis. All the assays are run in triplicate.

To measure cytotoxicity, targets are labeled with 100 μCi of ^{51}Cr for 60 minutes, plated in 96-well V-bottomed plates (3 x 10^3 cell/well), and pulsed with peptides (1 μM) at 37° C for 2 hours. Effectors are added and incubated at 37 °C for an additional 4 hours. One hundred μl of supernatant are collected, and the radioactivity is measured in a gamma counter. The percentage of specific lysis is determined as: (experimental release - spontaneous release)/(maximal release - Spontaneous Release) x 100.

As a surrogate marker for CTL responses, cytokine responses, such as IFN-γ (Mailliard et al., 2004, Cancer Res., 64:5934-37; Herr et al., 2000, Blood, 96:1857-64) and IL-2 (Carrabba et al., 2003, Cancer Res., 63: 1560-67) can be monitored. IFN-γ and IL-2 secretion levels from CTL cultures stimulated with native or superagonist peptides are measured using cytokine-specific ELISA and IFN-γ enzyme-linked immunospot assays.

The relative affinity (RA) of CD133 superagonist peptides for HLA-A*0201 are measured. Briefly, T2 cells are incubated with various concentrations of peptides ranging from 100 to 0.1 μM overnight and then stained with BB7.2 mAb to quantify the surface

expression of HLA-A*0201 allele. For each peptide concentration, the HLA-A*0201-specific staining is calculated as the percentage of staining obtained with 100 μM of the reference peptide HIVpol589 (IVGAETFYV; SEQ ID NO:25). The RA is determined as: RA = (concentration of each peptide that induces 20% of HLA-A*0201 expression / concentration of the reference peptide that induces 20% of HLA-A*0201 expression).

The stability of superagonist peptide/HLA-A*0201 complexes is assessed. Briefly, T2 cells are incubated overnight with 100 μM of each peptide. Cells are then incubated with Brefeldin A (Sigma, St. Louis, MO) at 10 μg/ml for 1 hour, washed, incubated at 37 °C for 0, 2, 4, or 6 hours in the presence of Brefeldin A (0.5 μg/ml), and then stained with BB7.2 mAb. For each time point, peptide induced HLA-A*0201 expression is calculated as: mean fluorescence of peptide preincubated T2 cells - mean fluorescence of T2 cells treated in similar conditions in the absence of peptide. DC50 is defined as the time required for the loss of 50% of the HLA-A*0201/peptide complexes stabilized at t = 0.

CTL are generated from human PBMCs. PBMCs are collected by leukapheresis from healthy HLA-A*0201 volunteers. Dendritic cells are produced from adherent cells (2×10^6 cells/ml) cultured for 7 days in the presence of 500 IU/ml granulocyte macrophage colony-stimulating factor (Leucomax; Schering-Plough, Kenilworth, New Jersey) and 500 IU/ml IL-4 (R&D Systems, Minneapolis, Minnesota) in complete medium [RPMI 1640 supplemented with 10% heat-inactivated human AB serum, 2 μM L-glutamine (Invitrogen) and antibiotics]. On day 7, dendritic cells are collected and pulsed with 40 μg/ml peptide in the presence of 3 μg/ml β2m (Sigma) for 4 hours at 20 °C and then irradiated (4200 rad). CD8+ T cells are isolated by positive selection with immunomagnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. A total of 0.5×10^6 CD8+ T cells are cocultured with 0.25×10^5 dendritic cells in a final volume of 0.5 ml/well in a 48-well plate in the presence of 10 ng/ml IL-7 (R&D Systems). Human IL-10 (R&D Systems) at 10 ng/ml is added the next day, and 30 IU/ml human IL-2 (Proleukin; Chiron Corp.) is added on day two. Seven and 14 days after the primary stimulation, CD8+ T cells are restimulated with irradiated adherent cells pulsed with 10 μg/ml peptide in the presence of 3 μg/ml β2m. Human IL-10 (10 ng/ml) and IL-2 (50 IU/ml) are added 24 and 48 hours later,

respectively. Seven days after the second restimulation, individual wells from the cultures are tested for peptide specific cytotoxicity on peptide loaded T2 cells in the presence of cold K562 cells (hot/cold target ratio 1:33 ratio).

CTL are also generated from glioblastoma patients. PBMCs from a total of 30 HLA-A2+ glioma patients are evaluated for their in vitro responsiveness against wild-type and superagonist peptides. The proportion of human patients that will develop specific CTLs capable of recognizing the wild-type CD133 peptide after stimulation with the superagonist peptide is determined. It is also determined whether these CTL recognize peptide-pulsed T2 cells or HLA-A2+ cancer stem cell lines that express CD133.

Intracellular production of IFN-γ is detected. A total of 5 x 10^4 T cells are incubated with 10^5 peptide-loaded T2 cells or with 10^5 tumor cells in the presence of 20 μg/ml Brefeldin A at 37 °C. Six hours later, the cells are stained with phycoerythrin-conjugated anti-CD8 mAb (Caltag Laboratories, Burlingame, California) in PBS for 25 minutes at 4 °C and fixed with PBS 4% Paraformaldehyde (Sigma). The cells are then permeabilized with PBS + 0.5% BSA + 0.2% saponin (Sigma) and stained with adenomatous polyposis coli-conjugated anti-IFN-γ mAb (PharMingen, Mississauga, Ontario, Canada) for 25 minutes at 4 °C. Cells are analyzed on a BD FACSCalibur™ flow cytometer (Becton Dickinson, Mountain View, CA).

Enzyme-linked immunosorbent spot (ELISPOT) assay kits (R & D Systems, Minneapolis, Minnesota) are used to detect immune responses. Responder (R) 1×10^5 patients' PBMC from before and after vaccination are plated in 96-well plates with nitrocellulose membrane inserts coated with capture Ab. Stimulator (S) cells (T2 pulsed potential peptide) are added at the R:S ratio of 1:1. After a 24-hour incubation, cells are removed by washing the plates 4 times. The detection Ab is added to each well. The plates will be incubated at 4 °C overnight, and the washing steps are repeated. After a 2-hour incubation with streptavidin-alkaline phosphatase, the plates are washed. Aliquots (100 μl) of BCIP/NBT alkaline phosphatase substrate solution are added to each well to develop the spots. The reaction is stopped after 60 minutes by washing with water. The spots are scanned and counted with computer-assisted image analysis (Cellular Technology Ltd, Cleveland, Ohio). When experimental values are significantly different

from the mean number of spots against non-pulsed T2 cells (background values), as determined by a two-tailed Wilcoxon rank sum test, the background values are subtracted from the experimental values. This assay provides a coefficient of variation of intra-assay for ELISPOT of less than 10%.

The superagonist-induced CTLs possess higher avidity, due to either higher affinity or stability between TCRs and peptide-MHC complexes. The higher avidity correlates with the avidity of T cell-target interactions and the antitumor responsiveness of T cells. The intensity (Yee et al., 1999, J. Immunol., 162:2227-34), or stability (Dutoit et al., 2002, J. Immunol., 168:1167-71) of specific T-cell staining with HLA tetramers, and threshold of positive staining using titrating doses of tetramers (Ercolini et al., 2005, J. Exp. Med., 201:1591-1602) are indicative of the relative avidity of specific T cells.

Example 8. Functional Assays of Immunogenic Peptides

This example demonstrates the usefulness of some of the HLA-A2 immunogenic peptides described herein in functional assays.

HLA-A2 positive PBMC isolated by leukapheresis from two healthy human subjects were obtained from HemaCare Corporation (Van Nuys, California). The PBMC from each subject were used to prepare CD8+ T cells and autologous dendritic cells for stimulation with immunogenic peptides. Following stimulation, the T cells were subjected to functional assays.

CD8+ T cells were prepared from each subject's PBMC by positive selection using CD8 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer's instructions.

Autologous DC were prepared from adherent PBMC (2 x 10^6 cells/ml) cultured for 5 days in the presence of 500 IU/ml granulocyte macrophage colony-stimulating factor (Leucomax; Schering-Plough, Kenilworth, New Jersey) and 500 IU/ml IL-4 (R&D Systems, Minneapolis, Minnesota) in complete medium [RPMI 1640 supplemented with 10% heat-inactivated human AB serum, 2 μM L-glutamine (Invitrogen) and antibiotics]. On day 5, DC maturation was induced by addition of 50 ng/ml TNF-α. On day 7, the mature DC were collected and pulsed with 20 μg/ml peptide for 4 hours at 20 °C. Peptide-pulsed DC not used immediately were frozen for later use.

The peptide pulsed matured DC were diluted to 3 x 10^5 cells/mL in CTL complete medium and irradiated at 2800 Rads. The initial in vitro stimulation was performed at a DC:T cell ratio of 1:5 by plating 100 µL/well of each batch of DC into a 96 well round bottom tissue culture plate (3 x 10^4 DC/well). One plate was prepared per peptide. To all wells, 100 µL of the CD8+ enriched T cell population was added at 1.5 x 10^6 cells/mL was added, resulting in a final concentration of 1.5 x 10^5 T-cells/well. Plates were incubated at 37 °C, 5% carbon dioxide, and high humidity.

On days 7 and 14, cryopreserved peptide pulsed mature DC were thawed and used to restimulate the T-cells. Cryopreserved samples of each peptide pulsed DC batch were removed from liquid nitrogen and thawed rapidly in a 37 °C water-bath. Samples were diluted at least 1:10 with AIM V medium (Invitrogen, Carlsbard, California). Samples were washed twice by centrifugation at 400 g for 7 minutes, and representative samples were evaluated for cell concentration and viability. Each DC batch was diluted to 3 x 10^5 cells/mL in CTL complete medium containing 20 U/mL IL-2, 10 ng/mL IL-7, and 2 µg/mL of the specific peptide, and the DC were irradiated with 2800 Rads. In vitro stimulation plates were removed carefully from the incubator and 90 µL of supernatant (no cells) were removed from each well. To each well, 100 µL of the appropriate DC batch was added. Plates were then incubated further at 37 °C, 5% carbon dioxide, and high humidity.

After the second in vitro stimulation, the plates were checked daily for T-cell proliferation. When required, plates were split (one plate will become two plates) to avoid overgrowth of the CTL. Generally, this occurred on days 19, 23, 26, and 30. The plates were mixed to ensure a homogenous population, then 90 µL of each CTL well was transferred into a new 96 well U bottomed plate. When splitting was performed within 3 days of the last in vitro stimulation, 100 µL of CTL complete medium containing 20 U/mL IL-2 and 10 ng/mL IL-7 was added. If splitting was performed later than 3 days from the last in vitro stimulation, 100 µL of complete CTL medium containing 40 U/mL IL-2 and 20 ng/mL IL-7 was added. Split plates were returned to the incubator at 37 °C, 5% carbon dioxide, and high humidity.

On day 21, the T cells were stimulated a fourth time. The process described for in vitro stimulations 2-3 was repeated with the exception that IL-2 was replaced with 25 ng/mL IL-15.

Stimulated CTL were assayed for staining using peptide-HLA-A2 dimers. Cells were collected 19 days after the third stimulation (subject 1) or 6 days after the fourth stimulation (subjects 1 and 2). Dimers of each peptide presented on HLA-A2 were prepared using BD™ DimerX I: Recombinant Soluble Dimeric Human HLA-A2:Ig Fusion Protein (BD Biosciences) according to the manufacturer's instructions at a 640-fold excess of peptide and 2 μg of HLA-A2:Ig protein. The HLA-A2:Ig fusion protein consists of three extracellular major histocompatibility complex (MHC) class I HLA-A2 domains that are fused to the VH regions of mouse IgG1. Immunofluorescence staining was performed essentially according to the manufacturer's instructions. The CTLwere washed and resuspended in FACS staining buffer. Non-specific binding was blocked with polyclonal human IgG. Peptide-loaded HLA-A2:Ig protein was added, and the samples were incubated for 60 minutes at 4 °C. The cells were washed and blocking solution of human IgG was added again. For immunofluorescence, FACS buffer containing PE-conjugated anti-mouse IgG1 was added. Following incubation at room temperature, the cells were fixed in 2% paraformaldehyde and analyzed by flow cytometry. The percentages of cells that stained positive (above background) for both dimer and CD8 are indicated in Table 7 below. SEQ ID NOs: 11 and 21 stained strongly above background, SEQ ID NOs: 28 and 29 stained moderately above background, and the SEQ ID NO:20 staining was indistinguishable from background.

Table 7. Peptide Dimer Staining

SEQ ID NO	Antigen	Sequence	Subject 1		Subject 2
			3rd stim	4th stim	4th stim
11	CD133	YLQWIEFSI	38.64	20.74	19.39
21	CD133	FLLPALIFA	20.43	13.23	21.18
28	CD133	ILSAFSVYV	4.15	0.12	14.08
20	CD133	GLLERVTRI	0.27	–0.41	–0.32

29	Mart-1a	ELAGIGILTV	0.50	0.82	0.68
		no peptide	NT	0	0.01

CTL were collected 6 days after the third and fourth stimulations for analysis by ELISPOT. Effector cells (5000 or 25,000 T cells stimulated with peptide-pulsed DC or DC without peptide) were plated in 96-well plates with nitrocellulose membrane inserts coated with anti-human IFN-γ Ab. Stimulator cells (1×10^5 peptide-pulsed DC) were added to each well. 5 μg/ml PHA was used as a positive control. After a 16-20-hour incubation, the cells were removed by washing the plates. A biotinylated anti-human IFN-γ detection Ab (Mabtech mAb 7-B6-1 Biotin) was added to each well, and the plates were incubated at 20 °C for 2 hours. After a 1-hour incubation with avidin-phosphatase complex (Vectastain ABC Elite), the plates were washed. Aliquots of 3-amino-9-ethylcarbazole (AEC) substrate (Vectastain AEC Kit) were added to each well to develop the spots. The reaction was stopped after 4-10 minutes (when spots appeared) by washing with water. The spots were inspected visually. Wells that clearly had more spots than the negative control were indicated as positive (Table 8). For subject 1, only Mart-1a was positive; for subject 3, both Mart-1a and SEQ ID NO:11 were clearly positive.

Table 8. ELISPOT Assay

SEQ ID NO	Sequence	Subject 1		Subject 2	
		3rd stim	4th stim	3rd stim	4th stim
11	YLQWIEFSI	–	–	–	++
21	FLLPALIFA	–	–	–	–
28	ILSAFSVYV	–	–	–	–
20	GLLERVTRI	–	–	–	–
29	ELAGIGILTV	–	++	++	++

This example demonstrates that the immunogenic peptides disclosed herein can stimulate induction of CTL.

OTHER EMBODIMENTS

A number of embodiments of the invention have been described. Nevertheless, it will be understood that various modifications may be made without departing from the spirit and scope of the invention. Accordingly, other embodiments are within the scope of the following claims.

WHAT IS CLAIMED IS:

1. An immunogen comprising an isolated peptide of 800 amino acid residues or fewer comprising the amino sequence of any one of SEQ ID NOs:1-21 with three or fewer amino acid substitutions.

2. An immunogen comprising an isolated peptide of 800 amino acids or fewer comprising a superagonist variant of any one of SEQ ID NOs:1-21.

3. A composition comprising the immunogen of claim 1 or 2.

4. The composition of claim 3, further comprising an adjuvant.

5. The composition of claim 4, wherein the adjuvant comprises complete Freund's adjuvant, incomplete Freund's adjuvant, Montanide ISA-51, LAG-3, aluminum phosphate, aluminum hydroxide, alum, or saponin.

6. A composition comprising the immunogen of claim 1 or 2 linked to an immunogenic carrier.

7. The composition of claim 6, wherein the immunogenic carrier comprises serum albumin, tetanus toxoid, keyhole limpet hemocyanin, dextran, or a recombinant virus particle.

8. The composition of claim 3, further comprising a cytokine.

9. The composition of claim 8, wherein the cytokine is IL-1, IL-2, IL-7, IL- 12, IL-13, IL-15, TNF, SCF, or GM-CSF.

10. The composition of claim 3, further comprising a vehicle.

11. The composition of claim 10, wherein the vehicle is a liposome, an immuno stimulating complex (ISCOM), or a slow-releasing particle.

12. The composition of claim 11, wherein the liposome comprises an emulsion, a foam, a micel, an insoluble monolayer, a liquid crystal, a phospholipid dispersion, or a lamellar layer.

13. The composition of claim 3, wherein the composition comprises antigen presenting cells.

14. The composition of claim 13, wherein the antigen presenting cells comprise dendritic cells.

15. Use of the immunogen of claim 1 or 2 or the composition of any of claims 3-14 for immunizing a subject.

16. The use of claim 15, wherein the subject has a cancer.

17. The use of claim 16, wherein cells of the cancer express CD133.

18. Use of the composition of any of claims 3-14 for the treatment of a cancer.

19. The use of claim 18, wherein cells of the cancer express CD133.

20. An in vitro method for inducing a cytotoxic T lymphocyte (CTL) that is specific for a cell expressing CD133, the method comprising contacting a precursor CTL with the immunogen of claim 1 or 2 under conditions that generate a CTL response to said tumor cells.

21. A method for preparing a cell vaccine for treating a cancer, the method comprising:

culturing bone marrow-derived mononuclear cells in a culture vessel under conditions in which the mononuclear cells can adhere to the vessel;

selecting a subset of the mononuclear cells comprising cells that have adhered to the vessel;

culturing the subset of mononuclear cells in the presence of one or more cytokines under conditions in which the subset of mononuclear cells differentiate into antigen presenting cells; and

culturing the antigen presenting cells in the presence of the immunogen of claim 1 or 2 under conditions in which the cells present the peptides on major histocompatibility class I molecules, thereby preparing a cell vaccine.

22. A nucleic acid molecule comprising a nucleic acid sequence encoding the immunogen of claim 1 or 2.

23. The nucleic acid of claim 22, further comprising an expression vector.

24. The nucleic acid of claim 23, wherein the expression vector is a plasmid or a nonreplicative viral vector.

25. A kit comprising the immunogen of claim 1 or 2.

26. A kit comprising the nucleic acid of claim 22.

```
Hsap MALVLGSLLLLGLCGNSFSGGQPSSTDAPKAWNYELPATNYETQDSHKAGPIGILFELVH 60
Ptro MALVLGSLLLLGLCGNSFSGGQPSSTDAPKAWNYELPATNYETQDSHKAGPIGILFELVH 60
Mmul MALVLGSLLLLGLCGNSFSGGQPSSTDAPKAWNYELPATNYETQDSHTAGPIGILFELVH 60
Rnor MALVFSVLLLLGLCGKMASGGQPAFDNTPGALNYELPTTEYETQDTFNAGIIDPLYQMVH 60
Mmus MALVFSALLLLGLCGKISSEGQPAFHNTPGAMNYELPTTKYETQDTFNAGIVGPLYKMVH 60
Cfam MALLLGFLLLLELCWDTSALGPLSSTKGSDGLEFELPATNYETKDSNQAGPISVLFQIVQ 60
Btau MALLLGFLLLLGLCEDTLSEELSSSGYRPDGLEFQLPPTSYQTSDSYDPGLAGFFFQIVR 60
     ***::. **** ** .  :    :    . . :::**.*.*:*.*:  .*  . ::::*:

Hsap IFLYVVQPRDFPEDTLRKFLQ-KAYESKIDYDKPETVILGLKIVYYEAGIILCCVLGLLF 119
Ptro IFLYVVQPRDFPEDTLRKVIQ-KAYESKIDYDKPETVILGLKIVYYEAGIILCCVLGLLF 119
Mmul IFLYVVQPRDFPEDTLRKVIQ-KARESKIDYDKPETLILGLKIIYYEAGIILCSVLGLLF 119
Rnor IFLNVVQPNDFPQDLVKKLIQ-KRFDISVDTKE---------VAIYEIGVLICVILGLLF 110
Mmus IFLSVVQPNDFPLDLIKKLIQNKKFDISVDSKEPEIIVLALKIALYEIGVLICAILGLLF 120
Cfam VFLQVVQPHPFPEDILRKILQ-KKFDFSTDYDK---------IIYYEIGIIICAVLGLLF 110
Btau FFVQIVQPNAFPEDILRKIIQ-KKFDLSKEYDKPENVVLTLKIIYYEIGIIICAALGLLF 119
     .*: :***. ** * ::*.:* *  : . : .:         :  ** *:::*  *****

Hsap IILMPLVGYFFCMCRCCNKCGGEMHQRQKENGPFLRKCFAISLLVICIIISIGIFYGFVA 179
Ptro IILMPLVGYFFCMCRCCNKCGGEMHQRQKENGPFLRKCFAISLLVICIIISIGIFYGFVA 179
Mmul IILMPLVGYFFCMCRCCNKCGGEMHQRQKENGLFLRKCFAISLLVICIIISIGIFCGFVA 179
Rnor IFLMPLVGFFFCMCRCCNKCGGEMHQRQKQNESCRRKCLAISLLLICLLMSLGIAFGFVA 170
Mmus IILMPLVGCFFCMCRCCNKCGGEMHQRQKQNAPCRRKCLGLSLLVICLLMSLGIIYGFVA 180
Cfam VILMPLVGFCFGLCRCCNKCGGEMHQRQKKNGAFLRKYFTVSLLVICIFISVGIIYGFVA 170
Btau VILMPLVGFFFCLCRCCNKCGGEMHQRQKKSGPFLKKYFTISLLVICVFISIGIIYGFAA 179
     ::******  * :****************:.    :* : :***:**:::*:**  **.*

Hsap NHQVRTRIKRSRKLADSNFKDLRTLLNETPEQIKYILAQYNTTKDKAFTDLNSINSVLGG 239
Ptro NHQVRTRIKRSRKLADSNFKDLRTLLNETPEQIKYILAQYNTTKDKAFTDLNSINSVLGG 239
Mmul NHQVRTRIKRSRKLADSNFKDLRTLLNETPEQIKYILAQYNTTKDKAFSDLNSINSVLGG 239
Rnor NQQTRTRIQRTQKLAESNYRDLRALLTEAPKQIDYILGQYNTTKNKAFSDLDSIDSVLGG 230
Mmus NQQTRTRIKGTQKLAKSNFRDFQTLLTETPKQIDYVVEQYTNTKNKAFSDLDGIGSVLGG 240
Cfam NHHLRTRIEKTRKLAESNLKDLRTLLIGTPAQINYVLSQYASTKEKAFSDLDNIKSLLGG 230
Btau NHYMRTNVEETRKLSESNLNDLRTLLNVVPGQIDYILDQFTLPKEKAFDDLDNINLLVGG 239
     *:  **.:: ::**:.** .*:::**  .* **.*:: *:  .*:*** **:.*  ::**

Hsap GILDRLRPNIIPVLDEIKSMATA-------------------------IKETKEALENMN 274
Ptro GILDRLRPNIIPVLDEIKSMATA-------------------------IKETKEALENMN 274
Mmul GILDRLRPNIIPVLDEIKSMATA-------------------------IKETKEALENMN 274
Rnor RIKGQLKPKVTPVLEEIKAMATA-------------------------IRQTKDALQNMS 265
Mmus RIKDQLKPKVTPVLEEIKAMATA-------------------------IKQTKDALQNMS 275
Cfam GIHDQLRPKVIPVLDDIKAMAEA-------------------------IKETREALLNVN 265
Btau SIYERLKPKVLPVLKDIKDLAEDGKGFFPPLVSPVGASVLKQAVFLTDMKTNRDTLVRMN 299
      *  :*:*:: ***.:** :*                            :: .:::* .:.
```

FIG. 1A

```
Hsap STLKSLHQQSTQLSSSLTSVKTSLRSSLNDPLCLVHPSSETCNSIRLSLSQLNSNPELRQ 334
Ptro STLKSLHQQSTQLSSSLTSVKTSLRSSLNDPLCLVRPSSEICNSIRLSLSQLNSNPELRQ 334
Mmul STLKSLHQQSTQLSSSLTSVKTSLRASLNDPLCSVRPSSETCNSIRLSLSQLNSNPELRQ 334
Rnor SSLKSLRDASTQLSTNLTSVRNSIENSLNSNDCASDPASKICDSLRPQLSNLGSNHNGSQ 325
Mmus SSLKSLQDAATQLNTNLSSVRNSIENSLSSSDCTSDPASKICDSIRPSLSSLGSSLNSSQ 335
Cfam NTLKELKMSTAQLNTSLSDVKRNLEQSLNDPMCSVPPVATTCNNIRMSLGQLDDNTNLGQ 325
Btau TVLTDMKQSSAQLRTSLRDVKTNMEQTLMDPQCSSPAAAPTCDSIRKSLSVLDGSANFDH 359
     . *..::  ::** :.* .*: .:. :* .  *   . :  *:.:* .*. *... :  :

Hsap LPPVDAELDNVNNVLRTDLDGLVQQGYQSLNDIPDRVQRQTTTVVAGIKRVLNSIGSDID 394
Ptro LPPVDAELDNVNNVLRTDLDGLVQQGYQSLNDIPDRVQRQTTTVVAGIKRVLNSIGSDID 394
Mmul LPSVDAELDKVNNVLRTDLDGLVQQGYQSLNDIPDRVQSQTKTVVAGIKRVLNSIGSDID 394
Rnor LPSVDRELNTVNDVDRTDLESLVKRGYMSIDEIPNMIQNQTGDVIKDVKKTLDSVSSKVK 385
Mmus LPSVDRELNTVTEVDKTDLESLVKRGYTTIDEIPNTIQNQTVDVIKDVKNTLDSISSNIK 395
Cfam LPSLDKQIDNINNVLQTDLSSLVQKGYKSFNDIPEMVQNQTTDIVSDVKRTLNSLGSDIE 385
Btau LPSLDGHITQLDGLLQTDLSGLVQKANESLSNIPEEVQNQTRDFISEFKKTLNSLQSDVK 419
     **.:* .:  :  : :***..**::.  ::.:**: :* **  .:  .*..*:*: *.:.

Hsap NVTQRLPIQDILSAFSVYVNNTESYIHRNLPTLEEYDSYWWLGGLVICSLLTLIVIFYYL 454
Ptro NVTQRLPIQDILSEFSVYVNNTESYIHRNLPTLEEYDSYWWLGGLVICSLLTLIVIFYYL 454
Mmul NVTQHLPIQNILSEFSVYVNNTESYIHRNLPTLEEYDSYWWLGGLVICSLLTLIVIFYYL 454
Rnor NMSQSIPVEEVLLQFSHYLNDSNRYIHESLPRVEEYDSYWWLGGLIVCFLLTLIVTFFYL 445
Mmus DMSQSIPIEDMLLQVSHYLNNSNRYLNQELPKLEEYDSYWWLGGLIVCFLLTLIVTFFFL 455
Cfam NMSEQIPIQDKLSDFIGYINDTETYIHRNLPTLEEYDSYRWLGGLIVCCLLTLIVVFYYL 445
Btau NISTKIPIQKTLSNFVRYINDSEDYILQYLPTMEECDSYRWLVCLVICCLLTLILIFYLL 479
     :::  :*::. *  .  *:*::: *: . ** :** *** **  *::* *****: *: *

Hsap GLLCGVCGYDRHATPTTRGCVSNTGGVFLMVGVGLSFLFCWILMIIVVLTFVFGANVEKL 514
Ptro GLLCGVCGYDRHATPTTRGCVSNTGGVFLMVGVGLSFLFCWILMIIVVLTFVFGANVEKL 514
Mmul GLLCGVCGYDRHATPTTRGCVSNTGGVFLMVGVGLSFLFCWILMIIVVLTFVFGANVEKL 514
Rnor GLLCGVFGYDKRATPTRRGCVSNTGGIFLMAGVGFSFLFCWILMILVVLTFVVGANVEKL 505
Mmus GLLCGVFGYDKHATPTRRGCVSNTGGIFLMAGVGFGFLFCWILMILVVLTFVVGANVEKL 515
Cfam GLMCGTFGYDRHATPTRRGCVSNTGGIFLMVGVGISFLFCWILMTIVVLTFVIGGNMEKL 505
Btau GLLCGTLGYDQKATPTRRGCVSNTGGLLLMVGVGLSFFFSWIIMTIVVLTFVTGGNMEKL 539
     **:**. ***::**** *********::**.***:.*:*.**:* :****** *.*:***

Hsap ICEPYTSKELFRVLDTPYLLNEDWEYYLSGKLFNKSKMKLTFEQVYSDCKKNRGTYGTLH 574
Ptro ICEPYTSKELFQVLDTPYLLNEDWEYYLSGKLFNKSKMKLTFEQVYSDCKKNRGTYGTLH 574
Mmul ICEPYTSKELFRVLDTPYLLNEDWEYYLSGKLFNKSEMKLTFQQVYSDCKKNRGTYGTLH 574
Rnor LCEPYENKKLLQVLDTPYLLNDQWQFYLSGILLKNPDINMTFEQVYRDCKRGRGVYATFQ 565
Mmus LCEPYENKKLLQVLDTPYLLKEQWQFYLSGMLFNNPDINMTFEQVYRDCKRGRGIYAAFQ 575
Cfam VCEPYQNRKLFQILDTPYLLNENWKYYLSGMVLDKPDINLTFEQVYSDCKENKGIYSTLK 565
Btau VCEPYRNKKLFQVLDTPYLLNEDWKYYLSGLVFNKPDINLTFEQVYSDCKENKGLYATLK 599
     :**** .::*:::*******:::*::**** ::.:..:::**:*** ***..:* *.:::
```

FIG. 1B

```
Hsap LQNSFNISEHLNINEHTGSISSELESLKVNLN-IFLLGAAGRKNLQDFAACGIDRMNYDS 633
Ptro LQNSFNISERLNINEHTGSISSELESLKVNLN-IFLLGAAGRKNLQDFAACGIDRMNYDS 633
Mmul LENSFDISDYLNINEHTASISSELESLKVNLN-IFLLGAAGRKSLQDFAACGIDRMNYDT 633
Rnor LENVFNITENFNIERLSEDIVKELEKLNVNIDSIELLDKTGRKSLEDFAQSGIDRINYSM 625
Mmus LENVVNVSDHFNIDQISENINTELENLNVNIDSIELLDNTGRKSLEDFAHSGIDTIDYST 635
Cfam LENTYNISEHLNIQEHARNLSNDFKNMNVNIDNIVLLDAAGRKNLMDFSSSGVDTIDYNV 625
Btau LDHIYNVSEQLNITKHTGDINSNLENMNIRIEDIELLDKTGMKTLMDLRSSGIDDIDYAA 659
     *::  :::: :** . : .: .:::.:::.:: * **. :* *.* *:  .*:* ::*

Hsap YLAQTGKSPAGVNLLSFAYDLEAKANSLPPGNLRNSLKRDAQTIKTIHQQRVLPIEQSLS 693
Ptro YLAQTGKSPAGVNLLSFAYDLEAKANSLPPGNLRNSLKRDAQTIKTIHQQRVLPIEQSLS 693
Mmul YLAQTGKSPAGVNLLSFAYDLEAKANSLPPGNLRNSLKRDAQTIKTIHQQRVLPIEQSLS 693
Rnor YLQEAEKPPTKVDLLTFASFLETEANQLPDGNLKQAFLMDAQNIRAIHQQHVPPVQQSLN 685
Mmus YLKETEKSPTEVNLLTFASTLEAKANQLPEGKPKQAFLLDVQNIRAIHQHLLPPVQQSLN 695
Cfam YLAEMGKTPTKVNLLSFADDLDTKANNLPQGSLKQSLKNNAQNLKTIHHGQVMPLEQSMS 685
Btau YLNATERSPTRVNLISFANNLRRKANQLPSGNLKTSLKSHTDTLTNIHENQVVPLQNSMN 719
     **    :.*: *:*::**  *  :**.** *. : ::  ..:.:  **.  : *:::*:.

Hsap TLYQSVKILQRTGNGLLERVTRILASLDFAQNFITNNTSSVIIEETKKYGRTIIGYFEHY 753
Ptro TLYQSVKILQRTGNGLLERVTRILASLDFAQNFITNNTSSVIIEETKKYGRTIIGYFEHY 753
Mmul TLYQSVKILQRTGNGLLERVNRILASLDFAQNFITNNISSVIIEETKKYRKTIIGYFEHY 753
Rnor SLKQSVWALKQTSSKLPEEVKKVLASLDSAQHFLTSNLSSIVIGETKKFGRTIIGYFEHY 745
Mmus TLRQSVWTLQQTSNKLPEKVKKILASLDSVQHFLTNNVSLIVIGETKKFGKTILGYFEHY 755
Cfam TINQSIKELQHKSSGLRVKVANILSSLDSAQDFLQTRISSVIVKESSKYGNMIIGYFEHY 745
Btau AMHQQMKGLQYRTSGLKVRVSTTIFFLNSTQDFLTSQLSEVVVEESKQFGNKIISYFERY 779
     :: *.:  *:   . *  .*   :  *: .*.*: .. * ::: *:.:: . *:.***:*

Hsap LQWIEFSISEKVASCKPVATALDTAVDVFLCSYIIDPLNLFWFGIGKATVFLLPALIFAV 813
Ptro LQWIEFSISEKVASCKPVATALDTAVDVFLCSYIIDPLNLFWFGIGKATVFLLPALIFAV 813
Mmul MQWIEFSISEKVASCKPVATALDTAVDVFLCSYIIDPLNLFWFGIGKATVFLLPALIFAV 813
Rnor LQWVLYAITEKMTSCKPMITAMDSAVNGILCSYVADPLNLFWFGIGKATMLLLPAVIIAI 805
Mmus LHWVFYAITEKMTSCKPMATAMDSAVNGILCGYVADPLNLFWFGIGKATVLLLPAVIIAI 815
Cfam LQWVKISITEQIAACKPVATALDSAVDVFLCSYIIDPMNLFWFGIGKATIFLLPAIIFAV 805
Btau LQWVEMAITQQFAACRPVATALDSAVNVFLCSYIVDPLNLFWFGVGKATILLLPAVIFAV 839
     ::*:  :*:::.::*:*: **:*:**: :**.*: **:******:****::****:*:*:

Hsap KLAKYYRRMDSEDVYDDVETIPMKNMENGNNGYHKDHVYGIHNPVMTSPSQH 865
Ptro KLAKYYRRMDSEDVYDDVETIPMKNMENGNNGYHKDHVYGIHNPVMTSPSQH 865
Mmul KLAKYYRRMDSEDVYDDVETIPMKNPSH------------------------ 841
Rnor KLAKYYRRMDSEDVYDDVETVPMKNLENGSNGYHKDHLYGVHNPVMTSPSRY 857
Mmus KLAKYYRRMDSEDVYDDVETVPMKNLEIGSNGYHKDHLYGVHNPVMTSPSRY 867
Cfam KLAKYYRRMDSEDVYDE----------------------------------- 822
Btau KLAKYFRRMYSEDVYEDEPVNKVQSKPRAQTVPRVQTVPMVQTVPMTKAL-- 889
     *****:*** *****::
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FIG. 1C